# Proteomic and phenotypic approaches to assess nutritional physiology in Atlantic salmon

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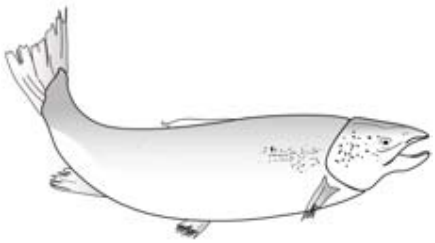

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The research associated with this thesis abides by the international and Australian codes on human and animal experimentation, the guidelines by the Australian Government's Office of the Gene Technology Regulator and the rulings of the Safety, Ethics and Institutional Biosafety Committees of the University. All research conducted for this thesis was approved by the University of Tasmania Animal Ethics Committee (Permit No. A0013044, A0014093, A0015208).

Signed,

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Hobart, September 12th, 2016

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**Paper 2,** Preliminary validation of a high docosahexaenoic acid (DHA) and alpha-linolenic acid (ALA) dietary oil blend: tissue fatty acid composition and liver proteome response in Atlantic salmon (*Salmo salar*) smolts. PLos One 11(8): e0161513. Located in Chapter 4.

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**DEDICATION**

To my grandparents:

Antonio Nuez

Celso Ortín

Paquita Martínez

Pilar Luño

*Por todos los inolvidables momentos que siempre llevo conmigo*

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**GENERAL ABSTRACT**

Atlantic salmon aquaculture increasingly faces global sustainability and environmental challenges that influence fish growth, nutritional value of the final product and production efficiency. Proteomics has established itself as an exploratory approach to understand the impact of changes in environmental factors and to production strategies at a detailed biological level. This thesis aims to examine the use of label-free shotgun proteomics as a method to further our understanding of current and important key aspects of Atlantic salmon aquaculture production: triploidy, fish oil replacement and heat stress.

Understanding diet- and environmentally-induced physiological changes in fish larvae is a major goal for the aquaculture industry. The application of proteomics to study larval fish is challenging due to the large dynamic range of protein expression in such complex biological matrix. Using sequential protein extraction to reduce sample complexity achieved proteome coverage of 40% greater than a conventional direct extraction method. Proteins and functional categories related to mitochondrial function, oxidative phosphorylation and antioxidant defense were particularly enriched, providing a platform for a better understanding of physiological changes.

A considerable knowledge gap exists regarding the physiology of triploid fish. A multi-tissue (whole fish, muscle and liver) and time-series proteomics sampling-approach was combined with fatty acid analysis to explore triploid-specific physiological and phenotypic traits of freshwater Atlantic salmon under optimal growing conditions. The very high level of similarity between the triploid and diploid fish at the proteome level was paralleled by subtle differences in growth and body composition. This is the first proteome characterization of freshwater triploid Atlantic salmon and supports the idea of physiological similarity between ploidies under optimal growing conditions.

The future use of novel docosahexaenoic acid (DHA)-enriched *Camelina* oil emerges as the most efficient and long-term strategy to reduce fish oil use in aquafeeds while improving the current nutritional value of salmon products for consumers. The formulation of an oil blend with similar fatty acid profile to DHA-enriched *Camelina* oil was combined with liver proteomics to understand tissue fatty acid deposition and the consequent dietary induced metabolic changes in Atlantic salmon smolt. This study demonstrated the suitability of this novel DHA-enriched oil to improve the fillet content of omega-3 long-chain (≥C20) polyunsaturated fatty acids (n-3 LC-PUFA) obtained with a current commercial blend oil. The liver proteome reflected lipid peroxidation-induced oxidative stress and the subsequent activation of the antioxidant and detoxification response, drawing attention to the need of additional antioxidant supplementation if DHA-enriched *Camelina* oil is used at high inclusion rates.

Heat stress causes concerns regarding negative effects on performance and health of fish. Liver proteomics was used to identify the underpinning mechanisms of adaptation to chronic elevated temperature (21°C) in pre-harvest Atlantic salmon. The induction of oxidative stress was paralleled by the suppression of the protein turnover rates, with the latter and an increased dependence on amino acid

catabolism as the main mechanisms to balance for the increased energy demand. This study corroborates candidate biomarkers of thermal stress and refines our understanding towards the development of salmon feeds for summer conditions.

In conclusion, the studies presented in this thesis advance our understanding of some of the key factors pertinent to Atlantic salmon farming globally, that are fundamental to growth and production efficiency, nutritional value and industry sustainability. Identification of molecular mechanisms and stressors in parallel with phenotypic changes underlines the potential of proteomics to further advance animal production generally and for aquaculture specifically, and provide focus for further targeted studies towards the development of specific nutritional and husbandry strategies. This work supports the current industry standards to produce high quality triploid smolt and suggests revision of the requirements of antioxidant, amino acid and other supplements in Atlantic salmon under the present sustainability and environmental challenges.

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**ABBREVIATIONS**

**2-DE**, two-dimensional electrophoresis

**AD**, apparent digestibility

**ALA**, α-linolenic acid (18:3n-3)

**DE**, direct extraction

**DF**, direct fraction

**DHA**, docosahexaenoic acid (22:6n-3)

**Dph**, days post-hatching

**DO**, dissolved oxygen

**EPA**, eicosapentaenoic acid (20:5n-3)

**FAMB**, fatty acid mass balance.

**FDR**, false discovery rate

**FER**, feed efficiency ratio

**FFA**, free fatty acids

**FL**, fork length

**FO**, fish oil

**FOPO**, blend of 20% fish oil and 80% poultry oil

**GC**, gas chromatography

**GC-MS**, gas chromatography mass spectrometric

**GE**, gross energy

**GO**, gene ontology

**GO_BP**, gene ontology biological process

**GO_CC**, gene ontology cellular compartment

**GO_MF**, gene ontology molecular function

**GSI**, whole gut-somatic index

**HIS**, hepato-somatic index

**IPA**, Ingenuity pathway analysis

**k**, Fulton's condition factor

**LA**, linoleic acid

**LC-MS/MS**, liquid chromatography-tandem mass spectrometry

**LFQ**, label-free quantification

**LTQ-Orbitrap**. linear trap quadruple-Orbitrap

**LW**, liver weight

**MDS**, multidimensional scaling

**MS**, mass spectrometry

**MS/MS**, tandem mass spectrometry

**MRM**, multiple reaction monitoring

**MUFA**, monounsaturated fatty acids

**PCSI**, pyloric caeca-somatic index

**OA**, oleic acid

**n-3**, omega-3

**n-3 LC-PUFA**, omega-3 long-chain polyunsaturated fatty acids

**n-3:n:6 ratio**, omega-3:omega-6 ratio

**n-6**, omega-6

**PCA**, principal component analysis

**PL,** phospholipids

**PUFA**, polyunsaturated fatty acids

**SE**, sequential extraction

**SEM**, standard error of the mean

**SC-PUFA**, shorter chain polyunsaturated fatty acids

**SF1**, sequential fraction #1

**SF2**, sequential fraction #2

**SGR**, specific growth rate

**ST,** sterols

**TAG**, triacylglycerols

**TOFX**, blend of 60% tuna oil and 40% flaxseed oil

**TW**, tissue weight

**W**, fish weight

**WE**, wax esters

**Wi**, initial weight

**Wf**, final weight

**Wg**, weight gain

# CHAPTER 1

# General introduction, aim and thesis structure

## 1.1. GENERAL INTRODUCTION

### 1.1.1. Introduction – three key aspects of salmon production

Global aquaculture has expanded at an average annual rate of 7.9% over the period of 1990 – 2012 and presently provides about 50% of all fish supplies destined for human consumption (The State of World Fisheries and Aquaculture, 2014). With an annual production growth rate of 9% since 1994, Atlantic salmon is placed as the leading intensively farmed marine fish and the second highest species in commercial value (Marine Harvest, 2015). Atlantic salmon has the highest level of industrilisation and the lowest risk among aquaculture products (Marine Harvest, 2015). Given the projected world population growth, global aquaculture production, including that of Atlantic salmon, will continue growing (World Bank, 2013).

Increased output requires further understanding and improvement of current production strategies aimed at global sustainability and environmental challenges. A strategy of increasing interest in salmon production is using triploids, which aims to mitigate the negative effects of early sexual maturation and to protect wild stocks from interbreeding with farmed fish (Benfey, 2015). Another strategy is sustainable feed formulation, which aims to reduce the use of wild-harvest fishery products (Naylor et al., 2009). In addition, aquaculture production is under the influence of ocean conditions and climate change, and enhancing our understanding of how Atlantic salmon cope with the increased surface seawater temperature is crucial to more accurately match production strategies to environmental conditions (Olsvik et al., 2013). Molecular biology techniques can complement the measurement of growth and body composition and provide an insight into physiological aspects not otherwise macroscopically evident. In this regard, proteomics is a non-hypothesis driven approach that can reveal unprecedented mechanisms that shape important commercial traits.

This introduction aims to highlight three key aspects relating to salmon production: 1) triploidy, 2) sustainable feeds, with specific focus on fish oil replacement, and 3) thermal stress; and to introduce exploratory proteomics as an approach to gain understanding of the metabolic changes induced by these three factors. It must be stressed that these factors are fundamental to the current production of Atlantic salmon in Tasmania (Australia). As a climate change 'hotspot' (Frusher et al., 2014), improved knowledge on fish physiology and how locally applied production strategies and environmental conditions affect Atlantic salmon can assist national and also global aquaculture in the future.

### 1.1.2. Triploid salmon – current application and the physiology knowledge gap

Triploid fish are reproductively sterile, and their relevance in aquaculture principally relates to the increasing need for protecting wild stocks from the interbreeding that can occur following escapes (Benfey, 2015). Triploid Atlantic salmon is currently farmed only in Tasmania, where it represents 30-40% of the total harvest (Tassal Tasmanian Salmon, personal communication). In this region, however, the main goal of triploid farming is to avoid the negative effects on growth, immune-competence and

flesh quality associated with early sexual maturation (Sadler, 2009). Tasmanian salmon has a greater rate of early maturation due to the higher water temperatures that occur compared to other production regions (Porter et al., 2005), and triploid farming fills the harvest gap derived from precocious stock and ensures appropriately sized fish are available for harvest all year round (Benfey, 2009, Sadler, 2009). The Tasmanian industry uses all-female triploids, also avoiding the changes in flesh quality associated with hormonally competent triploid males (Lincoln and Scott, 1984, Benfey, 1999, Sadler et al., 2000).

Artificial triploidization is commonly induced by hydrostatic pressure shock and provides the cell nucleus with three sets of chromosomes as opposed to the usual two (Preston et al., 2013). As a consequence, triploid fish are composed of larger, but fewer, cells than in the diploid counterpart (Piferrer et al., 2009). It has been suggested that this condition elicits physiological responses that are responsible for deleterious effects on cultured fish (Maxime, 2008). These are referred as the major bottlenecks in triploid salmon production and include the poor tolerance to stress from sub-optimal environments (McCarthy et al., 1996, Atkins and Benfey, 2008, Hansen et al., 2015), a high incidence of skeletal deformities (Leclercq et al., 2011, Fjelldal et al., 2012, Amoroso et al., 2016a) and cataract development (Wall and Richards, 1992, Taylor et al., 2015). Other attributes of triploid salmon relative to diploids include fewer pyloric caeca and shorter guts (Peruzzi et al., 2014), increased heart and brain weight (Fraser et al., 2012, Fraser et al., 2015), less aggressive feeding in communal rearing (Carter et al., 1994, Taylor et al., 2014) and differing gut microbiota composition (Cantas et al., 2011). These attributes of triploid salmon have the potential to affect performance traits and further suggest altered physiological status relative to diploids. Whether they are derived from a physiological condition generated by increased cell size, and whether increased cell size interferes with physiological mechanisms involved in growth and nutrient utilization, is unknown.

Triploid performance has been evaluated in a number of studies and recently reviewed (Benfey, 2015). Recent improvements in the performance of triploids have been achieved using triploid-specific nutrition (Taylor et al., 2015, Fjelldal et al., 2016) and husbandry (Fjelldal et al., 2012, Fraser et al., 2013) strategies. Nonetheless, considering the increasing interest in triploid production and the knowledge gap about their physiology (Maxime, 2008, Alcántar-Vázquez, 2016), it is important to further explore specific physiological and phenotypic traits of triploid Atlantic salmon under all production conditions. Identifying underlying mechanisms that explain differences in key phenotypic traits of Atlantic salmon, such as growth and fatty acid composition, is crucial for the further development of triploid specific production strategies. In this regard, the freshwater stage offers a physiological window to gain specific insight into the ploidy effect derived from differences in cell physiology and to avoid the influence of maturation related signals. This information will complement recent research by the Aquaculture group at the Institute for Marine and Antarctic Studies (IMAS) on deformities of triploid Atlantic salmon (Amoroso et al., 2016a, Amoroso et al., 2016b) and will be

valuable for the Tasmanian aquaculture industry in the first instance, and also globally, towards improving the efficiency and sustainability of Atlantic salmon production.

### 1.1.3. Use of alternate oil sources – novel docosahexaenoic acid (DHA)-containing oils to improve the actual nutritional value of salmon

The need to investigate alternate oil sources for fish oils is an industy goal and is the basis for a considerable amount of research effort towards the development of sustainable aquafeeds (Glencross, 2009, Miller et al., 2010, Turchini et al., 2013, Tocher, 2015). Sustainable feed formulation practices for Atlantic salmon routinely use alternative oils from land-based plant (e.g. canola) and animal (e.g. poultry oil, beef tallow) origin, with the latter being a common prcatice in Australia for the past decade (Codabaccus et al., 2012, Emery et al., 2016). These oils, containing predominantly monounsaturated fatty acids (MUFA) and shorter chain (SC, $\leq C_{18}$) polyunsaturated fatty acids (SC-PUFA) to varying degrees, are normally blended with fish oil at inclusion rates that do not compromise salmon productivity (Nichols et al., 2014).

Producing salmon in a sustainable manner also implies safeguarding its nutritional value for consumers (Garnett et al., 2013), which is largely provided by its high content in protein and omega-3 long-chain (LC, $\geq C_{20}$) polyunsaturated fatty acids (n-3 LC-PUFA). Reports from Australia and Europe show that the fillet content of n-3 LC-PUFA has dropped by over half during the last decade (Figure 1.1) (Nichols et al., 2014), and is highly variable among retailers (Henriques et al., 2014), which has been atttributed by the same authors to the above-mentioned industry feeding practices.

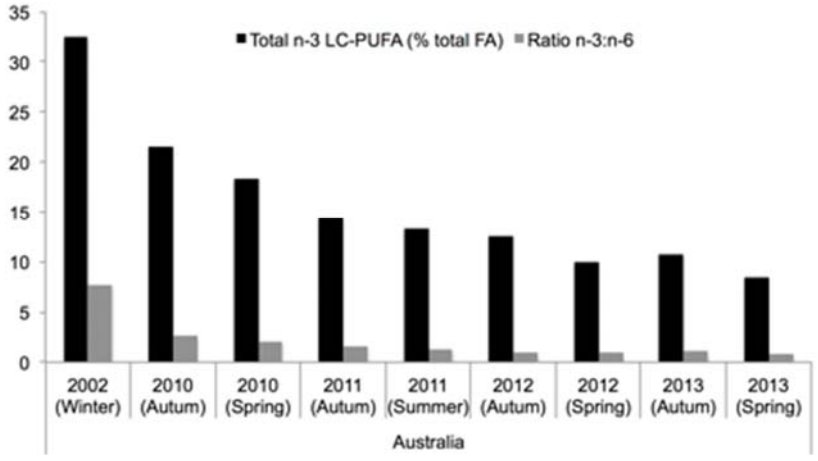


**Figure 1.1. Decline in the n-3 LC-PUFA composition (as % total FA) and n-3:n-6 ratio in fillet of Atlantic salmon sampled in Australia during the 2002-2013 period.** Adapted from Nichols et al. (2014).

As the aquaculture sector continues to grow, the increasing cost and fluctuations in the availability of fish oil will increase the difficulty to maintain the n-3 LC-PUFA content of salmon products (Henriques et al., 2014). As pointed out by Henriques et al. (2014), "this is a concern for the consumer as the health benefits of 'n-3' are increasingly being appreciated by the public and become an influential factor in fish consumption". Therefore, and despite the improvements of the aquaculture industry with the development of sustainable aquafeeds, a long-term solution to fish oil replacement is still required.

Novel oils extracted from transgenic oilseed crops containing n-3 LC-PUFA arise as a possible long-term strategy to reduce marine resource inputs (Kitessa et al., 2014). Production of n-3 LC-PUFA-containing oils is encouraged by their relatively low cost as well as by the potential capability to adequately scale up for large volume production and then applications (Petrie and Singh, 2011). The two oilseed crop species identified as potential hosts for the n-3 LC-PUFA biosynthetic trait are *Camelina sativa* and canola; both oil are characterized by high oil yield, are rich in MUFA and α-linolenic acid (18:3n-3, ALA), and show strong agronomic performance (Vollmann and Eynck, 2015). As an example given by Napier et al. (2015), 200,000 ha of transgenic *Camelina*, representing less than 3% of the land used for vegetable oil production in Canada, could produce 150,000 MT of oil, which could potentially replace 15% of the total oceanic harvest of fish oils. Parallel research has been conducted at Commonwealth Scientific and Industrial Research Organization (CSIRO) (Australia) and Rothamsted Institute (United Kingdom) to successfully produce a higher plant source of n-3 LC-PUFA. The former organization has extracted two different oils; one from *Camelina* containing ~12% of the total fatty acids as docosahexaenoic acid (22:6n-3, DHA), matching bulk fish oil levels, and ~29% ALA (Petrie et al., 2014), and one from canola containing ~13% DHA (Petrie et al., 2013) and similar levels of ALA to the *Camelina* also occurring (CSIRO, unpublished data, Nichols et al. 2016). The latter research team has produced two oil varieties from *Camelina*; one containing ~20% of total fatty acids as eicosapentaenoic acid (20:5n-3, EPA) (Ruiz-Lopez et al., 2014), and a second one containing DHA and EPA as 5% and 6% of the total fatty acids (Betancor et al., 2016), respectively . While these oil profiles clearly represent a long-term alternative to fish oil and are nutritionally attractive for the aquafeed industry, only commercial production of the CSIRO-developed DHA-containing oils is currently planned and is dated for release in 2018 (Nuseed, 2016). Therefore, validation of using a high DHA and ALA containing oil in aquafeeds to replace fish oil, and to enhance the nutritional value of salmon fillet, in relation to currently used commercial blend oils is required. Furthermore, this unusual fatty acid profile may induce metabolic changes in fish, and it is important to understand these changes for the future potential use in salmon aquafeeds of novel DHA-containing oil sourced from transgenic oilseeds. This information will supplement previous collaborative research between IMAS and CSIRO to validate alternative oils and to understand the physiological mechanisms underpinning dietary oil manipulation in Atlantic salmon (Miller et al., 2007b, a, Miller et al., 2008, Codabaccus et al., 2011, Codabaccus et al., 2012).

### 1.1.4. Thermal stress – towards developing strategies to cope with heat stress

Climate change is introducing several environmental challenges for fisheries and aquaculture (De Silva and Soto, 2009). Given the higher warming rate of the Southern Ocean, ectotherms species in this area are more vulnerable to climate change (Neuheimer et al., 2011). The east coast of Tasmania is a major growing area for Atlantic salmon, holding approximately 50% of the total production (Battaglene et al., 2008). During the summer period (January-April) of 2014, the sea surface temperature in eastern Tasmania reached an average temperature over 19°C (Figure 1.2). While these temperatures represent an increase of approximately 1°C in relation to the average temperature over the 1993-2013 period, a further 2°C increase over the entire region is projected for 2060 (Oliver et al., 2014).

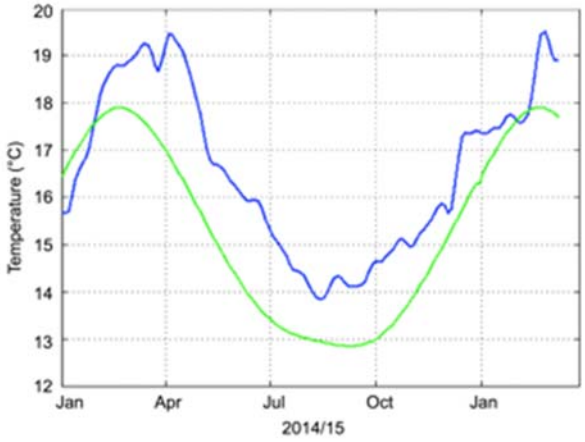


**Figure 1.2. Average sea surface temperature off Eastern Tasmania.** Average temperature over the 2014 - 2015 (blue line) and 1993 - 2013 (green line) periods. Temperatures over 19 °C were reached between March and May 2014. Image courtesy of IMOS OceanCurrent.

Seawater Atlantic salmon are generally considered to have an optimum temperature range of 13-15°C for growth and an upper critical range of 22-33°C (Jonsson and Jonsson, 2009). It is therefore evident that Atlantic salmon, under Tasmanian production conditions, is exposed to long periods of heat stress, raising both productivity and welfare challenges (Battaglene et al., 2008). These include reduced growth and feed conversion efficiency (Handeland et al., 2008), reduced content of n-3 LC-PUFA in muscle (Miller et al., 2006), early maturation (Porter et al., 2005), and increased risk of amoebic gill disease outbreak and infection (Douglas-Helders et al., 2001).

Tasmania, as a climate change hotspot, has a tradition of research on the physiology and nutrition of aquaculture production species in relation to elevated water temperature. Previous IMAS research has determined the optimum dietary protein to energy ratio as well as the maintenance energy and protein requirements of Atlantic salmon at elevated temperatures (Carter et al., 2008). The

interaction between the aquafeed fatty acid profile and temperature on fish lipid metabolism has been described in a variety of species including Atlantic salmon (Miller et al., 2006, Ng et al., 2010, Alhazzaa et al., 2013, Codabaccus et al., 2013), and has contributed towards the identification of alternate oils sources more suitable for elevated temperatures. Biochemical and molecular tools (e.g. targeted gene expression analysis) have been used in Atlantic salmon and barramundi, the latter as a model species, to gain understanding of the effect of temperature on regulating protein synthesis and degradation mechanisms (Katersky and Carter, 2007b, Carter et al., 2008, Katersky and Carter, 2010). This information has benefitted the industry towards the improvement of aquafeed formulations better targeting the growing conditions during the summer period (Carter et al., 2008). Further developments are expected in relation to the capacity of more advanced non-targeted molecular techniques to explore and to gain further understanding of the mechanisms driving acclimation of Atlantic salmon to elevated temperature. Given the demonstrated specificity of the thermal stress response to exposure regime (Logan and Buckley, 2015), generation of commercially relevant data can provide insight on the chronic temperature exposure as is typically encountered in Tasmania during the summer period. This information and the subsequent adaptation of production strategies are important locally, but also build-up a platform to deal with climate change at the global level in the future.

### 1.1.5. Shotgun proteomics - an exploratory tool to gain physiological insight into triploidy, dietary oil manipulation and thermal stress

Proteomics is commonly referred to as the large-scale study of a specific proteome, which represents the final and stable product of many redundant gene expression processes (Boersema et al., 2015). Unlike the transcriptome, the proteome covers the complex nature of gene expression, accounting for post-transcriptional and post-translational regulation of protein expression, and thus capturing relevant information missed by the transcriptome (Schwanhäusser et al., 2011). Proteomics requires no priori assumptions regarding which pathways or processes might be affected, and thus, if complemented with phenotypic data, stands as a viable and useful exploratory approach to investigate fish physiology.

Most proteomics workflows rely on database searching in which experimental peptide mass spectra are scored against theoretical mass spectra derived from a generic protein database. A problem with protein database searching is that proteins of fundamental biological significance, but whose exact sequences are missing from the generic databases to which they are matched, remain undetected. A solution to this detection problem is the use of genomic or transcriptomic nucleotide sequencing data to create customized or augmented proteomic databases for MS-based proteomics database searching (Altschul et al., 1990, Camacho et al., 2009). For Atlantic salmon, a high-quality assembly and annotation of whole genome has been made recently available (Lien et al., 2016), while the application of high-throughput technologies to survey RNA, especially microarray profiling and RNA sequencing

(RNAseq), have contributed to the generation of tissue-specific transcript sequences (Tacchi et al., 2012, Evans et al., 2015, Valenzuela-Miranda et al., 2015).

Quantitative proteomic approaches can be classified as either gel based or gel free methods as well as "label-free" or "label-based". While a detailed description of each approach has been well reviewed (Abdallah et al., 2012, Deracinois et al., 2013, Zhang et al., 2013), this section limits to provide a definition of the concept as well as an illustrative description (Figure 1.3) of the gel-free, label-free shotgun approach used in the following research chapters. Shotgun proteomics provides an indirect measurement of proteins through analysis of peptides derived from the proteolytic digestion of intact proteins (Zhang et al., 2013). The traditional shotgun approach is liquid-phase chromatography (LC) procedures coupled with high-resolution tandem mass spectrometry (MS/MS) without prior protein gel separation (Zhang et al., 2013). Since peptides can be more easily separated than proteins by liquid chromatography, the peptide-based proteomics approach generally allows for a larger dynamic range of quantification than the gel-based approach (Baggerman et al., 2005). Label-free quantitation refers to the non-chemical or -metabolic labeling of proteins, and it is largely employed due to its rapidity, low cost and simplicity of use in relation to label-based techniques (Deracinois et al., 2013).

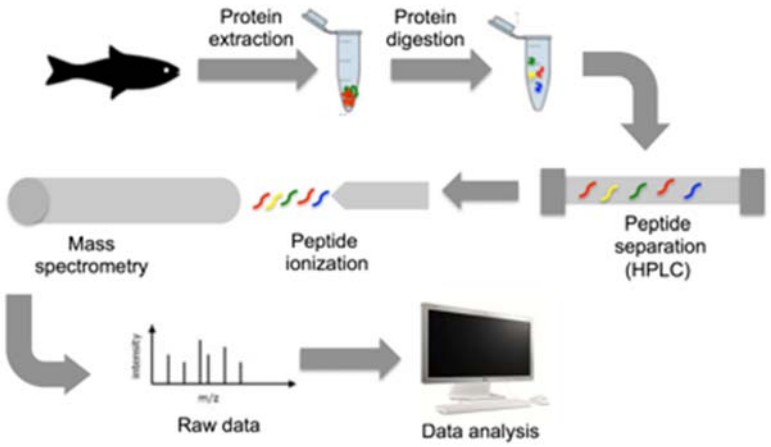


**Figure 1.3. Generic shotgun proteomics workflow used in this thesis.** Samples are first homogenized in buffer solution and protein is extracted. The protein solution is digested with a protease, usually trypsin, into peptides. These are eluted from a liquid chromatography column and introduced into the mass spectrometer by electrospray ionization. Peptide masses are recorded and MS/MS spectra of the peptide daughter ions are searched against a protein database to determine the peptide sequences and then to identify the protein.

As reviewed (Rodrigues et al., 2012, Zhou et al., 2012), proteomics has been widely applied in aquaculture research, habitually in species of higher commercial importance and covering the areas of nutrition, welfare, product quality, and disease. These studies have provided not only a better understanding of mechanisms of nutrient utilization, pathogenesis or toxicity, but also information on candidate biomarkers for stress, flesh quality or disease. Gel-based proteomics has so far been the

standard approach in most aquaculture studies, while the application of the shotgun analysis is more recent (Timmins-Schiffman et al., 2013, Valdenegro-Vega et al., 2014, Piovesana et al., 2016) and shows a general increase in protein identification rates relative to the gel approaches. Given the proven capability of proteomics in aquaculture research, this thesis will use shotgun proteomics to explore the physiological response of Atlantic salmon to triploidy, dietary oil manipulation and thermal stress. As outlined above, these variables induce changes in growth and body composition. Ultimately, a better understanding of the mechanisms underpinning these phenotypic responses at the proteome level may provide fundamental insights into the respective adaptation processes of Atlantic salmon and further assist towards the improvement in growth efficiency, product quality and industry sustainability in Tasmania.

### 1.1.6. Protein fractionation – towards improving proteome coverage in aquaculture research

One of the greatest challenges in proteomics research is the large dynamic range of protein expression (Zhang et al., 2013). Identification and quantification is biased towards high abundance proteins and the effective dynamic range is limited to a restricted order of magnitude, limiting the depth of proteome coverage. This problem is particularly challenging in complex protein mixtures such as tissues dominated by highly-abundant structural components (e.g. cartilage, muscle) (Wilson et al., 2010, Deshmukh et al., 2015), biofluids (e.g. plasma) (Hortin and Sviridov, 2010), or whole organisms (e.g. whole fish larvae) (Gómez-Requeni et al., 2010). A number of protein and peptide fractionation technologies have been developed to deal with the large dynamic of protein abundance, aiming to reduce sample complexity and the subsequent impact of under sampling (i.e. defined as when only a fraction of the detectable peptides are identified because the total number of peptides eluting from the LC column per unit time exceeds the analytical capacity of the mass spectrometer) (Wang et al., 2010). Ultimately, reduced sample complexity improves peptide detection and the robustness of protein identification and coverage. As comprehensively reviewed (Wu and Han, 2006, Lee et al., 2010, Doucette et al., 2011), different fractionation strategies can be applied in a shotgun proteomics set-up. These include subcellular fractionation, solubility-based protein extraction, immuno-affinity depletion of known highly abundant proteins, selective equalization of the protein dynamic range using combinatorial ligand libraries, or advanced chromatographic separation. Desirable qualities in a fractionation strategy are the degree of orthogonality, to achieve maximal separation with the minimum number of fractions in a time and cost-efficient manner, and reproducibility, the latter for enhancing reliable and robust protein identification and quantitation (Wilson et al., 2010). However, for any given application, not all approaches are available or equally suitable for a cost- and time-effective analysis. Depletion strategies are costly and might result in non-specific binding, and more advanced chromatographic separation technologies require extensive method development and specific instrumentation (Zhang et al., 2013), while subcellular fractionation is labor-intensive and biased towards specific organelles (Lee et al., 2010). The application of sequential protein extraction in progressively denaturing buffers was firstly applied for the extraction of membrane proteins in bacteria

(Molloy et al., 1998), and has more recently resulted in a time- and resource-effective strategy for proteomics analysis of structurally complex tissues such as cardiac muscle (Barallobre-Barreiro et al., 2013), cartilage (Wilson et al., 2010) and bone (Jiang et al., 2007). Inter-sample reproducibility and extensive proteomic profiling was efficiently achieved by the separation of intracellular proteins, membrane proteins and tightly interacting matrix components.

There is increasing interest in the application of proteomics in aquaculture research, including the characterization of complex biological matrices such as muscle (Mohanty et al., 2015, Piovesana et al., 2016) and whole fish larvae (Sveinsdottir and Gudmundsdottir, 2011, Maneja et al., 2014, Chicano-Galvez et al., 2015). In the analysis of both types of samples, protein identification is hampered by the large dynamic range of protein abundance, and particularly by the abundant structural components. While direct protein extraction in one denaturing buffer has been the standard extraction method used for whole fish larvae and muscle in aquaculture studies, the sequential extraction method can contribute to improving proteome coverage by reducing sample complexity through the separation in different fractions of structural components from those readily soluble. Within the context of this thesis, the efficiency of sequential protein extraction was first validated as sample fractionation method for whole fish larvae, then used in combination with shotgun proteomics to investigate triploidy-induced physiological changes in whole fish larvae and muscle.

## 1.2. THESIS AIM and STRUCTURE

The overall aim of this thesis is to gain physiological insight into three key aspects of Atlantic salmon production in Tasmania that are fundamental to fish growth and production efficiency, product quality and industry sustainability: triploidy, fish oil replacement, and thermal stress. This has been approached by examining fundamental phenotypic production and body composition parameters for Atlantic salmon and by implementing a shotgun proteomics approach to explore the underlying physiological mechanisms. This aim has been addressed in four research chapters that have been prepared as stand-alone manuscripts and have been published or are in preparation for submission to scientific journals.

**Chapter 2** validated the efficiency of the sequential protein extraction method for improved proteome coverage of whole fish larvae in relation to the standard direct extraction methods [Nuez-Ortín. W. G., Carter, C. G., Nichols, P. D., Wilson, R. (2016). Sequential protein extraction as an efficient method for improved proteome coverage in larvae of Atlantic salmon (*Salmo salar*). Proteomics. DOI: 10.1002/pmic.201600051]

**Chapter 3** examined the impact of triploidy on growth, body composition and proteome response of Atlantic salmon at different developmental intervals during freshwater rearing and under optimal growing conditions. The proteomes of whole fish alevin as well as of muscle and liver tissues of fry and parr fish were characterized. For the proteomics analysis of complex biological matrices,

such as whole fish larvae and muscle, the sequential extraction method was applied aiming to achieve extensive proteome coverage.

**Chapter 4** examined the use of a formulated oil blend with a high DHA and high ALA content, as found in DHA-containing oils from transgenic *Camelina sativa* and/or canola, on tissue fatty acid deposition and the liver proteome response of Atlantic salmon smolt in relation to fish oil and a commercial oil blend [Nuez-Ortín. W. G., Carter, C. G., Cooke, I., Wilson, R., Nichols, P. D., (2016). Preliminary validation of a high docosahexaenoic acid (DHA) and alpha-linolenic acid (ALA) dietary oil blend: tissue fatty acid composition and liver proteome response in Atlantic salmon (*Salmo salar*) smolts. PLoS One. DOI: 10.1371/journal.pone.0161513]

**Chapter 5** examined the impact of long-term exposure to heat stress (21°C) on the liver proteome response and biometric indices of pre-harvest Atlantic salmon. Proteomics analysis was performed on an individual fish basis, rather than pooling as in the previous chapters, with this approach aiming to increase statistical power for the detection of fold changes.

A general discussion (**Chapter 6**) summarising the main findings and implications of this thesis and providing recommendations for future research directions is also presented.

# CHAPTER 2

# Sequential protein extraction as an efficient method for improved proteome coverage in larvae of Atlantic salmon (*Salmo salar*)

Published as Nuez-Ortin, W. G., Carter, C. G., Nichols, P. D. and Wilson. R. 2016. Sequential protein extraction as an efficient method for improved proteome coverage in larvae of Atlantic salmon (*Salmo salar*). Proteomics 16(14):2043-2047.

## 2.1. ABSTRACT

Understanding diet- and environmentally-induced physiological changes in fish larvae is a major goal for the aquaculture industry. Proteomic analysis of whole fish larvae comprising multiple tissues offers considerable potential, however, is challenging due to the very large dynamic range of protein abundance. To extend the coverage of the larval phase of the Atlantic salmon (*Salmo salar*) proteome, we applied a two-step sequential extraction (SE) method, based on differential protein solubility, using a non-denaturing buffer containing 150 mM NaCl followed by a denaturing buffer containing 7 M urea and 2 M thiourea. Extracts prepared using SE and one-step direct extraction (DE) were characterized via label free shotgun proteomics using nanoLC-MS/MS (LTQ-Orbitrap). SE partitioned the proteins into two fractions of approximately equal amounts, but with very distinct protein composition, leading to identification of ~40% more proteins than DE. This fractionation strategy enabled the most detailed characterization of the salmon larval proteome to date and provides a platform for greater understanding of physiological changes in whole fish larvae.

Note: This chapter is written in a continuous style as per Technical briefs published in Proteomics.

## 2.2. DESCRIPTION

Aquaculture is the fastest growing animal-food producing sector, with Atlantic salmon (*Salmo salar*) among the highest value and volume species (World Bank, 2013). The production of high quality salmon larvae is a key target of commercial aquaculture because the larval phase is a critical period that influences further predispositions during the remaining life cycle (Carter, 2015). Proteomics can be applied to enhance our understanding of physiological responses to environmental and dietary factors that determine larval quality (Chicano-Galvez et al., 2015). However, as with any tissue or organism comprising a highly complex mixture of cellular proteins and tissues dominated by abundant structural components, depth of proteome coverage is hampered by the very large dynamic range of protein abundance. Protein and/or peptide fractionation can offer a partial solution, but must be tested empirically in the biological system being analyzed. Desirable qualities in a fractionation strategy are orthogonality, to achieve maximal separation per fraction, and reproducibility, for robust protein identification and quantitation. While fractionation of proteins based on differential solubility has been used effectively to reduce sample complexity prior to two-dimensional electrophoresis (2-DE) analysis (Wilson and Bateman, 2008), it is not commonly used in shotgun proteomics studies. Proteomics-based analysis of commercial fish larvae has generally utilized 2-DE after direct protein extraction in denaturing buffer (Rodrigues et al., 2012) but this approach falls short in terms of reproducibility and detection of lower-abundance proteins (Chicano-Galvez et al., 2015). Here, we used label-free quantitative proteomics to determine the effectiveness of sequential extraction (SE) based on differential protein solubility as an effective strategy to increase proteome coverage of whole fish salmon larvae.

Eight *Salmo salar* larvae (98 days post-hatched; average weight of 150 mg) were sampled prior to first feed from an experiment at the Institute for Marine and Antarctic Studies, University of Tasmania (Launceston, Tasmania, Australia) in accordance with University animal ethics (Investigation A0013044). Fish were euthanized (50 mg/L, Aqui-S$^{TM}$), immediately snap-frozen and stored at -80°C prior to protein extraction. SE involved homogenization of four individual larvae, each in 3 ml ice-cold non-denaturing buffer (150 mM NaCl, 50 mM Tris, pH 8.0) containing protease inhibitor cocktail (Roche) using a Tissue-Tearor homogenizer (Biospec Products, OK, USA). Samples were rotated overnight at 4°C then centrifuged at 13,000 rpm (15 mins at 4°C) to obtain sequential fraction SF1. The salt-insoluble fraction was re-suspended and rotated overnight at 4°C in 1.5 ml denaturing buffer (7 M urea, 2 M thiourea, 50 mM Tris, pH 8.0) containing protease inhibitor cocktail, then centrifuged as above to obtain sequential fraction SF2. To evaluate the SE method, we compared it to single-step direct extraction (DE) of four larvae, each homogenized directly in 3 ml ice-cold denaturing buffer, followed by mixing and centrifugation as above to obtain a single protein fraction (DF). Aliquots of SF1, SF2 and DF (200 µl) were precipitated with 100% ethanol (9:1, v/v) and pellets washed twice in 70 % ethanol. Extracts were re-suspended in denaturing buffer to 1 µg/µl as determined by Bradford

assay (Bio-Rad). The protein yields by DE and SE were ~15 mg and ~18 mg per larvae, respectively, with 52% and 48% of SE protein partitioning between extracts SF1 and SF2, respectively.

Protein samples were trypsin-digested using standard procedures (Wilson et al., 2010) and analyzed by nanoLC-MS/MS using an LTQ-Orbitrap XL and Ultimate 3000 nanoHPLC system (ThermoFisher Scientific, MA, USA). Tryptic peptides (~1 µg) were loaded onto a 20 mm x 75 µm PepMap 100 trapping column (3 µm $C_{18}$) at 5 µl/min, using 98% water, 2% acetonitrile and 0.05% TFA. Peptides were separated at 0.3 µl/min on a 250 mm x 75 µm PepMap 100 RSLC column (2 µm $C_{18}$) at 40ºC from 97% mobile phase A (0.1% formic acid in water) to 50% mobile phase B (0.08% formic acid in 80% acetonitrile and 20 % water) by elution with 3-10% B over 10 min, 10-40% B over 120 min, 40-50% B over 10 min, holding at 95% B for 10 min then re-equilibration in 3% B for 15 min. SE peptide extracts were injected once and DF extracts were injected twice to achieve equivalent analysis time. The LTQ-Orbitrap was controlled using Xcalibur 2.1 software in data-dependent mode as described (Wilson et al., 2016).

MS/MS spectra were searched against the Salmonidae database (http://uniprot.org/taxonomy/8030; 17,795 entries) using the Andromeda search engine in MaxQuant, version 1.5.1.2 (http://maxquant.org/). Default settings for protein identification by LTQ-Orbitrap MS/MS and label-free quantitation (LFQ) included a maximum of two missed cleavages, mass error tolerances of 20 ppm then 4.5 ppm for initial and main peptide searches, respectively, 0.5 Da tolerance for fragment ions, variable methionine oxidation and fixed cysteine carbamidomethylation. The false discovery rate (FDR) for both peptide-spectrum matching and protein identification was set to 0.01. To compare protein identification rates by SE and DE, RAW files for the two methods were processed independently, where SF1 and SF2 were defined as sample fractions, as were replicate DF injections. To compare sequential extracts, SF1 and SF2 were defined as different experimental treatments. MaxQuant peptides.txt and proteinGroups.txt output files are provided (Supplemental Tables 2.1 - 2.4). Perseus software (http://perseus-framework.org) was used to compare sample groups and for statistical analysis. Proteins only identified by site, reverse sequences, contaminants and proteins identified on the basis of a single matching peptide were excluded.

To assess the benefits of SE for whole fish proteomics, we first compared peptide and protein identification rates to DE for proteins detected in at least three biological replicates in any one sample group. The total peptide-spectra matches recorded for SE and DE were similar (21,789 *vs* 22,614, respectively). However, SE achieved a marked improvement in the number of unique peptides and protein groups identified, specifically a 44% increase in the number of unique peptide sequences relative to DE (3,613 *vs* 2,536, respectively), which were assigned to 740 and 538 protein groups by SE and DE, respectively. This ~40% improvement in proteome coverage included 298 proteins only identified using SE, while 68 proteins were identified only in the DE samples. Figure 2.1A shows the distribution of proteins identified according to the number of unique peptides. Notably, the SE approach

achieved higher identification rates across the full range of matched peptides. Furthermore, SE resulted in protein identification on the basis of more unique peptide matches for the ~500 proteins detected by both extraction methods (Figure 2.1B). Principal component analysis indicated a high level of consistency between replicate samples (Appendix Figure 2.2) and Pearson's correlation coefficients ranged between 0.965-0.983 for SE and 0.978-0.993 for DE.

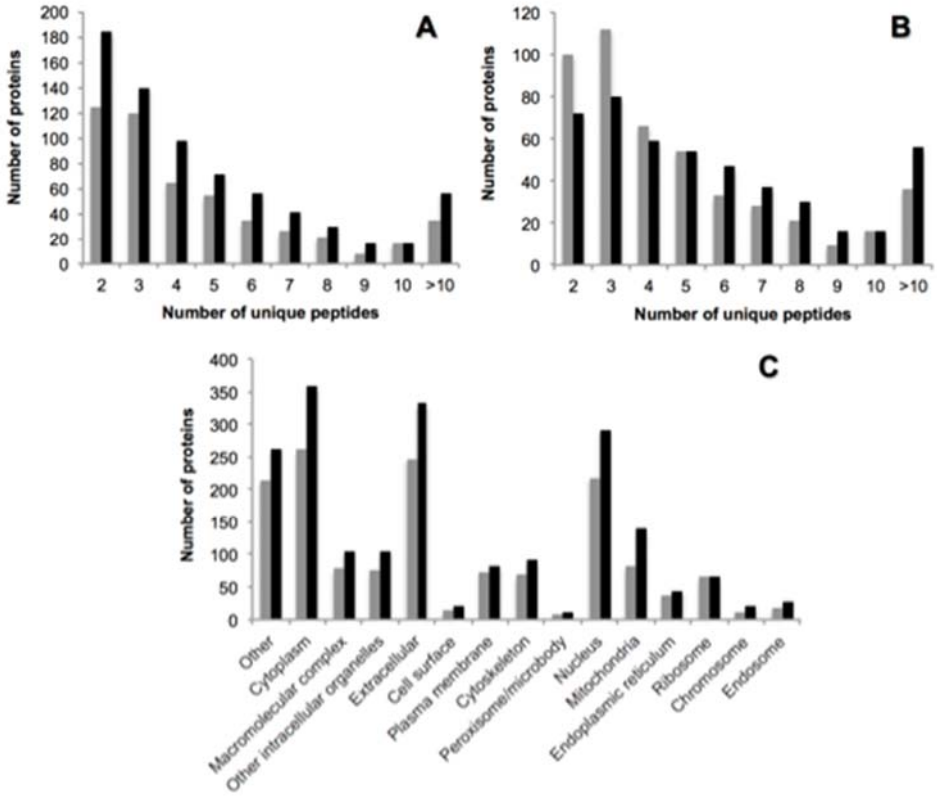


**Figure 2.1. Improvements in matched peptides/protein and organelle proteome coverage by sequential extraction (SE) of Salmo salar larvae compared with direct extraction (DE).** Bar diagrams display differences between DE (grey) and SE (black) in the frequency of protein identification according to: (A) the number of unique peptides for the respective 538 and 740 identified proteins; (B) the number of unique peptide matches for the 496 proteins detected by both extraction methods; (C) subcellular distribution based on STRAP analysis of human orthologues (355 and 512 genes by DE and SE, respectively).

To investigate the source of improved proteome coverage achieved using SE, we first used STRAP (Bhatia et al., 2009) to evaluate the subcellular distribution of the proteins identified, based on the Salmonidae proteins we could map to human orthologues (Mi et al., 2013). The improvement in protein identification was not universal, but was greatest for proteins assigned to mitochondrial, cytoplasm and extracellular categories, with 72%, 38% and 34% more proteins associated with these compartments, respectively (Figure 2.1C). Secondly, nearly twice the number of significant GO_MF

terms (adjusted $p$ values < 0.05) were identified using SE compared with DE (55 *vs* 27, respectively) according to the output from DAVID (Huang da et al., 2009) bioinformatics software (Supplemental Table 2.5). Consistent with a marked improvement in identification of mitochondrial proteins, SE-specific terms included "NADH dehydrogenase activity" (14 proteins), "oxidoreductase activity" (16 proteins), "monocarboxylic acid binding" (12 proteins), "peroxiredoxin activity" (6 proteins), "antioxidant activity" (10 proteins), and "ATPase activity, coupled to transmembrane movement of ions" (11 proteins). This enrichment of proteins and functional categories related to mitochondrial function, oxidative phosphorylation and antioxidant defence is particularly important in the context of fish larvae physiology, given the critical role of these processes in adaptation to their changing environment and diet (Bermejo-Nogales et al., 2015).

To further examine the improvement in proteome coverage we observed using SE, groups of proteins that were significantly enriched in SF1 and SF2 were identified. Peptide LFQ values, normalized according to the MaxLFQ algorithm using a minimum peptide ratio count of two (Cox et al., 2014),were first $\log_2$-transformed and proteins identified in fewer than three biological replicates were excluded. After imputation of remaining missing values with random intensity values for low-abundance proteins, mean LFQ values (SF2 *vs* SF1) were compared using a two-sided regularized *t*-test based on a stabilization factor of 1 and permutation-based FDR cut-off of 0.05. The results of each data processing step are presented in Supplemental Tables 2.6 – 2.8. Remarkably, 639 of the 701 proteins considered were significantly enriched in one fraction, with fold-differences between mean LFQ values ranging from 1.5-50x (enriched in SF1) and 1.6-718x (enriched in SF2). To identify functionally-related proteins, the complete set of significantly enriched proteins was sorted alphanumerically (Supplemental Table 2.9) and protein groups were highlighted on volcano plots (Figure 2.2). Using this approach, it was readily apparent that SE effectively partitioned groups of freely-soluble cytosolic constituents (e.g. 14-3-3 proteins; plot A) from large, multi-subunit protein complexes (e.g. ribosomal subunits; plot C). The SE method also revealed separation of related proteins on the basis of intermolecular interactions. For example, the SF2-enriched eukaryotic translation initiation complex subunits 1-3, that are ribosome-bound in the 43S complex (des Georges et al., 2015), were separated from the SF1-enriched subunits 4-5 involved in transient and weaker mRNA capping/de-capping interactions (plot G). Similarly, the alpha/beta core subunits of the 26S proteasome (SF1-enriched) were completely separated from the regulatory subunits (SF2-enriched), consistent with previous analysis of mouse cartilage using sequential extraction with 1 M NaCl and 4 M GuHCl (Wilson et al., 2010). In contrast to cartilage, which is dominated by extracellular proteoglycan and fibrillar collagen networks, the most highly SF2-enriched proteins were myosin polypeptides and regulatory chains (plot H), reflecting the abundance of skeletal, smooth and cardiac muscle sub-types in salmon larvae.

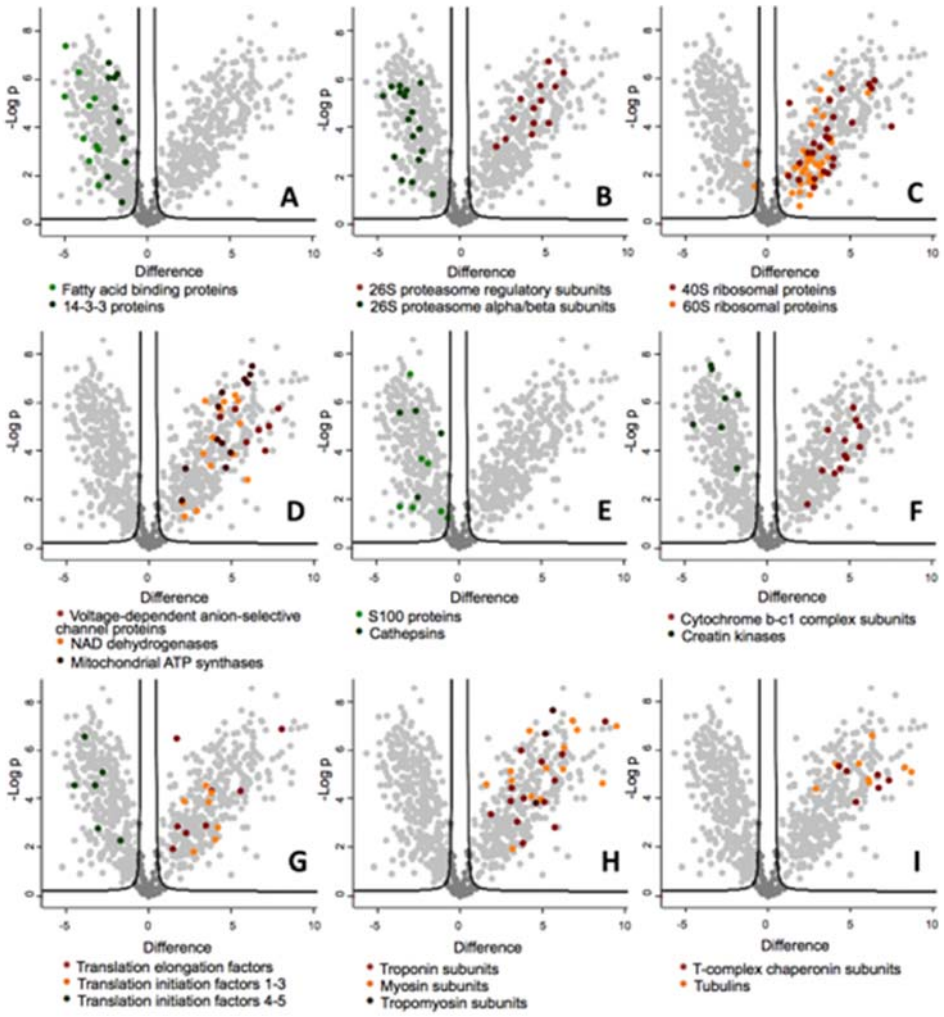


**Figure 2.2. Protein functional groups enriched in sequential extracts of *Salmo salar* larvae.** Volcano plots display the results of *t*-test comparison of fractions SF1 and SF2. Highlighted data points represent significantly enriched and functionally-related proteins.

To further characterize the SE protein fractions, the ~450 significantly enriched proteins that could be mapped to human orthologues were submitted to DAVID to identify functional terms and pathways associated with SF1 and SF2 (Supplemental Table 2.10 and 2.11, respectively). Although this comparison was not based on the complete set of differentially abundant proteins, significant gene ontology (GO) terms directly related to all of the protein groups displayed in Figure 2.2 were retrieved. Functional terms associated with the SF2-enriched proteins included GO_BP "translation", comprising both ribosomal subunits (plot C) and initiation/elongation factors (plot G), GO_CC "sarcomere" and GO_BP "skeletal muscle contraction" including troponins and myosins (plot H) and the Interpro terms "proteasome subunit P45" (plot B) and T-complex "chaperonin TCP-1, conserved site" (plot I). The GO_CC term "mitochondrial part" included respiratory protein complexes such as the ATPase subunits, NAD dehydrogenases, voltage-dependent anionic channels (plot D) and cytochrome C oxidase and b-

C1 complex subunits (plot F). DAVID analysis also identified additional SF2-enriched mitochondrial proteins associated with the inner and outer mitochondrial membranes such as the import receptor TOMM22, the inner membrane translocase subunit TIMM13 and components of the fatty acid beta oxidation pathway (ACADM, HADA and HADB). Significant functional terms associated with SF1-enriched proteins included GO_MF "S100 beta binding" (plot E), the KEGG pathway "PPAR signaling" representing fatty acid binding proteins (plot A), GO_BP "regulation of apoptosis" which included several 14-3-3 proteins (plot A), GO_MF "peptidase activity" comprising alpha and beta proteasome subunits (plot B), GO_CC "vacuole" which included the lysosomal cathepsins (plot E) and GO_BP "creatine metabolic process" (plot F). Additionally, DAVID analysis identified a major group of ~20 enzymes involved in energy metabolism, including many components of the glycolytic and gluconeogenesis pathways (GO_BP "monosaccharide metabolic process").

On the basis of these results, biochemical fractionation of salmon larvae using SE generates very distinct protein pools and in particular separates large protein complexes and structures from readily soluble components. We have shown that SE is an efficient approach to reduce protein sample complexity for whole fish proteomics, and has potential as the first step towards more extensive sample fractionation, for example at the peptide level. Beyond the improvement in peptide identification and proteome coverage, this approach could also be used, for example, to deplete highly abundant structural proteins or, conversely selectively enrich other protein groups of interest. The SE approach described will facilitate proteomic studies of whole fish larvae, leading to a more comprehensive understanding of the physiological impact of factors affecting production and quality in salmon and other commercial species.

The mass spectrometry data are available via the ProteomeXchange Consortium PRIDE partner repository, dataset PXD003366.

# CHAPTER 3

# Triploid Atlantic salmon (*Salmo salar*) shows similar performance, fatty acid composition and proteome response to diploids during early freshwater rearing

Nuez-Ortin, W. G., Carter, C. G., Nichols, P. D., Wilson, R., Cooke, I. R., Amoroso, G. and Cobcroft, J. M. 2016. Triploid Atlantic salmon (Salmo salar) shows similar performance, fatty acid composition and proteome response to diploids during early freshwater rearing. *In preparation.*

## 3.1. ABSTRACT

There is currently renewed interest in farming triploid Atlantic salmon. Improving farming requires identifying triploid specific phenotypic and physiological traits that are uniquely derived from ploidy *per se* and developed under optimal growing conditions. This study investigated firstly, the impact of ploidy on growth performance and whole body composition of Atlantic salmon at different early freshwater stages (34 dph (days post-hatching) alevin; 109 dph; fry and 162 dph parr;) and secondly, whether phenotypic differences at these stages were reflected in protein samples collected from whole fish, white muscle or liver tissue. Female diploid and triploid Atlantic salmon (n = 3) were first fed at 35 dph and then maintained by feeding to satiation on commercial feeds. Triploids were significantly lower in weight at the late alevin and fry stages but matched diploid weight at the parr stage. The whole-body lipid content was significantly higher for triploids at the parr stage, while the whole-body lipid class profile was generally broadly similar and was largely not affected by ploidy. Comparative label-free shotgun proteomic analysis did not detect significant alterations in protein expression between diploids and triploids at any growth stage. The present results indicate that ploidy under optimal growing conditions and during early freshwater stages only result in small phenotypic differences in weight and whole body lipid content that were not reflected at the proteome level. These findings suggest that optimal husbandry conditions for freshwater Atlantic salmon are similar between ploidies, at least for all-female populations.

## 3.2. INTRODUCTION

Triploidy in commercial aquaculture refers to the artificially induced condition of having three complete sets of chromosomes in the cell nucleus instead of two, rendering the triploid organism reproductively sterile. In the case of Atlantic salmon aquaculture, triploids are used to mitigate the negative impact on somatic growth and immune-competence associated with gonadogenesis, and to enhance industry sustainability by preventing interbreeding with wild populations following escapes (Jobling et al., 2010, Benfey, 2015). In addition, in some regions triploid salmon fill the harvest gap derived from early maturing stock and ensure appropriately sized harvest fish all year round (Benfey, 2009, Sadler, 2009). At the present time, triploid Atlantic salmon are only commercially produced in Tasmania (Australia), while Norway has recently approved the production of the first triploid population (Reuters, 2016) and commercial evaluation is underway in other European countries (Benfey, 2015). More precise understanding of triploid-specific phenotypic and physiological traits along the production cycle will allow the potential benefits of farming triploid Atlantic salmon to be better realised.

The feasibility of triploid salmon for commercial production is dictated by the life history during the freshwater stage (Johnston et al., 2003, Macqueen et al., 2008). Alterations during the early stages of development might permanently influence organism metabolism and growth potential (Reynolds and Caton, 2012). In addition, early growth stages offer a window to explore basic ploidy effects derived from potential differences in cell physiology or associated with the trauma caused by triploidization, in the absence of hormonally driven effects due to suppressed gonadal development. While these ploidy effects can potentially be reflected in differential expression of phenotypic traits such as growth or body composition, the underlying physiological mechanisms during early development have been poorly explored to date. Improved triploid juvenile performance was previously linked to reduced rates of protein degradation, however, this was observed under sub-optimal feeding conditions (i.e. re-feeding following feed deprivation) (Cleveland and Weber, 2013). Differences in energy and fatty acid utilization have also been suggested (Ozório et al., 2012, Sacobie et al., 2015), while detailed assessment of liver lipid metabolism as affected by ploidy has been provided during the period of sexual maturation (Manor et al., 2012, Manor et al., 2014, Manor et al., 2015, Cleveland and Weber, 2016) but is still lacking during earlier periods free of maturation-related signals. Further research to gain insight into the physiological effects of ploidy during early growth stages and under optimal growing conditions is critical for understanding the impact of ploidy *per se* on phenotypic traits such as growth and body composition and to identify alterations that can potentially affect these traits in later stages.

Proteomics is considered a powerful exploratory tool towards providing a better understanding of fish physiology. The proteome of highly metabolically active tissues, such as white muscle and liver, as well as that of whole fish larvae, is sensitive to dietary, environmental and developmental changes

(Forne et al., 2010, Rodrigues et al., 2012, Zhou et al., 2012) and therefore can potentially reflect differences in the regulation of growth or nutrient utilization as affected by ploidy. Shotgun proteomics, as opposed to traditional gel-based approaches, is still in its infancy in aquaculture research (Timmins-Schiffman et al., 2013, Zhang et al., 2013, Valdenegro-Vega et al., 2014, Piovesana et al., 2016). A recent technical development in shotgun proteomics of whole fish larvae utilized sequential extraction based on differential protein solubility to reduce sample complexity and significantly improve peptide identification and proteome coverage (Nuez-Ortin et al., 2016).

The first aim of the present study was to investigate the effect of ploidy on performance and body composition of early freshwater Atlantic salmon. The second aim was to use proteomics to identify altered patterns of protein expression in whole fish larvae, white muscle and liver in response to ploidy state. In order to achieve extensive proteome coverage, the analytical strategy chosen was solubility-based sequential protein extraction followed by label-free quantitative shotgun analysis. In particular, we tested the hypothesis that ploidy differences in growth and body composition under optimal growing conditions would reflect in the alteration of molecular pathways underlying nutrient metabolism. This study is the first to use a proteomics approach to examine triploid-specific physiological traits in freshwater Atlantic salmon. These findings will be of value for the aquaculture industry in the further development of triploid specific husbandry and nutritional strategies to achieve more effective and sustainable triploid farming.

## 3.3. MATERIALS and METHODS

### 3.3.1. Fish stock and experimental set-up

The experiment was conducted from July (winter) to January (summer) at the Institute for Marine and Antarctic Studies, University of Tasmania (Launceston, Tasmania, Australia) in accordance with University of Tasmania Animal Ethics (A0013044). A total of 7800 female diploid and triploid alevins (n = 3900 per ploidy) of Atlantic salmon at 14 days post-hatching (dph) were used in this experiment. This study was run in parallel with another study assessing the effect of ploidy on the prevalence of skeletal anomalies (Amoroso et al., 2016a), in which details of the protocols for fertilization, triploidization, and incubation were provided. During incubation, weak embryos were removed and a temperature of approximately 8 °C (7.8 ± 0.2 °C), as recommended (Fraser et al., 2013), was used to ensure timely and efficient egg development while avoiding high temperature-induced deformities.

Fish were transferred and allocated in replicate plastic crates (n = 650 per replicate) of 40 x 20 cm and 27 L capacity each. A total of 12 replicate crates were distributed in three square tanks of 2.2 x 2.2 m each and 4000 L capacity. Crates were placed on one side of the tank, opposite to inflow and with alternate allocation of diploid and triploid fish. Each tank was connected to its own recirculating system equipped with heat-chiller and filter. De-chlorinated freshwater was exchanged in each tank at 1 L min$^{-}$

$^{1}$ and delivered to each replicate crate by an airlift system consisting of an elbow-shaped pipe with a suspended airstone. Exchange rate in each crate was 1.5 L $min^{-1}$ with three exchange rates per hour. Airlift systems were identically assembled in order to match flow rates across the replicate crates. Stocking density ranged from 4.1 to 50 kg $m^{-3}$ between the beginning and the end of rearing in the crates. At 126 dph, fish were transferred to mesh cages of 90 x 50 x 50 cm within the same tanks. Stocking density ranged from 4.8 to 12.8 kg $m^{-3}$ between the beginning and the end of the mesh cage rearing phase.

Water temperature and photoperiod followed an identical regime as established by the industry. Temperature was maintained at 8°C until one week prior to the start of first feeding and gradually increased until reaching 10°C at first feeding (35 dph - 42 dph). Then, temperature was increased to 11°C one week after first feeding, and again gradually increased to 14°C over the course of two weeks and maintained until the end of the experiment (162 dph). Fish were exposed to 24 h light photoperiod. Water temperature was recorded every 15 min, while water quality parameters including dissolved oxygen, pH, nitrite, nitrate and chlorine, were recorded weekly and maintained within limits for Atlantic salmon (Wedemeyer, 1996). Mortality was assessed on a daily basis and dead fish were removed.

### 3.3.2. Growth trial and sampling

An overview of the experimental design, including sampling times, is illustrated in Figure 3.1. Fish were fed two commercial feeds (Skretting, Tasmania, Australia) following standard production protocols; fry feed (Nutra XP; Crude protein: 61.0 ± 1.02 g $kg^{-1}$ DM; Gross energy: 23.4 ± 0.10 MJ $kg^{-1}$) was fed from first feeding (35 dph - 42 dph) to 109 dph, followed by a parr feed (Nutra RC; Crude protein: 53.4.0 ± 0.49 g $kg^{-1}$ DM; Gross energy: 23.1 ± 0.08 MJ $kg^{-1}$) until the end of the experiment (162 dph). Dietary phosphorus concentrations (18.3 and 13.7 g $kg^{-1}$ DM in fry and parr feeds, respectively) were over the minimum requirement (9.4 – 16.3 g $kg^{-1}$ DM) recently recommended to support optimal performance and a low incidence of skeletal deformities in freshwater triploid Atlantic salmon (Fjelldal et al., 2016). Feeds were hand-fed to approximately meet satiation at a feeding rate of 8 and 3 times $day^{-1}$ during the fry and parr stages, respectively (Amoroso et al., 2016a).

The sampling events were determined by the above dietary adjustments that followed industry standards. At 34 dph (late stage alevin one day before the start of first feeding), 20 fish from each tank and ploidy were euthanized (AQUI-S: 50 mg $L^{-1}$) (Javahery et al., 2012), measured for individual weight and fork length, frozen in liquid nitrogen, and kept at -20 °C for carcass composition analysis. Four fish were alike euthanized, frozen in liquid nitrogen, and kept at -80 °C for further proteomic analysis. At 109 dph (9 weeks on the fry feed) and 162 dph (8 weeks on the parr feed), 20 fish from each tank and ploidy were sampled for carcass composition analysis. In addition, liver and dorsal white muscle from four fish per ploidy and tank were dissected out, weighed, immediately frozen in liquid nitrogen, and kept at -80°C until further proteomic analysis. Sampled fish were visually free of skeletal deformities and short opercula. Fish were fasted the day before sampling to evacuate their gut. Prior to

measuring, all excess water was removed by blotting with a paper towel. All dissecting procedures were performed on ice and completed within five min.

A 100% efficiency of triploid induction was verified at the end of the experiment by measuring erythrocyte diameter of 120 fish per ploidy (Amoroso et al., 2016a).

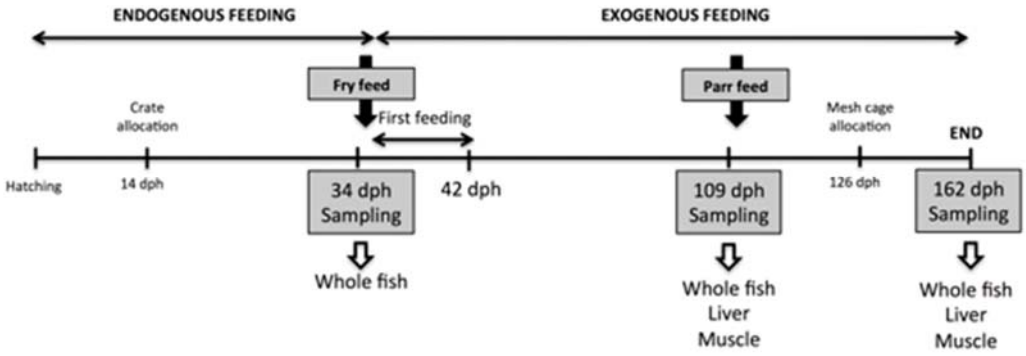


**Figure 3.1. Overview of the experimental design.** Fish were allocated in crates at 14 dph (days post-hatching) and moved to mesh cages at 126 dph. Growth was monitored from 22 dph to 162 dph. Samplings for analyses included 34 dph (before start of first feeding), 109 dph (9 weeks on the fry feed and before the start of parr feed) and 162 dph (8 weeks on the parr feed and end of the experiment).

### 3.3.3. Chemical composition

Whole bodies from each sampling time were pooled by tank, freeze-dried, and milled to a fine powder. Dry matter was obtained by drying at 135°C for 2 h and ash content after incineration at 600°C for 2 h (AOAC, 1995). Crude protein was calculated after determination of total nitrogen (Elemental analysis using EA 1112 Series Flash Elemental Analyser, Thermo Finnigan), based on N x 6.25 (AOAC, 1995). Total lipid was obtained following overnight extraction using a modified Bligh and Dyer protocol (Bligh and Dyer, 1959, Alhazzaa et al., 2011), involving a single phase extraction using dichloromethane/methanol/water (1:2:0.8, v/v/v) followed by phase separation to yield a total lipid extract. Gross energy was measured by bomb calorimeter (6725 Semimicro, Parr, IL, USA). All analyses were corrected for dry matter.

### 3.3.4. Lipid class and fatty acid analysis

Lipid class composition was determined after spotting of total lipid on silica rods (SIII), solvent development and then detection by an IatroscanTM MK-5 thin-layer chromatography-flame ionisation detector analyser (Mitsubishi Kagaku Iatron, Inc., Japan) (Alhazzaa et al., 2011). Peak areas were quantified by SIC-480II for IatroscanTM Integrating Software v.7.0-E (System Instruments Co., Mitsubishi Chemical Medience Corporation, Japan). The detector was calibrated for each class using a mixture containing wax esters (WE), triacylglycerols (TAG), free fatty acids (FFA), sterols (ST) and phospholipids (PL) (Sigma-Aldrich).

An aliquot of the total lipid extract was trans-methylated in methanol/dichloromethane/hydrochloric acid (10:1:1, v/v/v) at 80 °C for 2 h. After addition of mQ water (1 mL), the mixture was extracted with hexane/dichloromethane (4:1, v/v) three times to obtain fatty acid methyl esters (FAME). FAME were made up to a known volume with internal injection standard (19:0 FAME, Nu-Chek Prep, Inc., MN, USA) and analysed by a 7890B gas chromatograph (Agilent Technologies, California, USA) equipped with a Supelco Equity$^{TM}$-1 fused silica capillary column (15 m × 0.1 mm i.d., 0.1 μm film thickness), flame ionization detector, split/splitless injector, and a 7683B auto sampler (Agilent Technologies, CA, USA). Helium was used as the carrier gas and samples were injected in splitless mode at an oven temperature of 120 °C. After injection, oven temperature was increased to 270 °C at 10 °C min$^{-1}$ and to a final temperature of 300 °C at 5 °C min$^{-1}$. Peaks were quantified with ChemStation software (Agilent Technologies, CA, USA) and initially identified using retention times from authentic and laboratory standards. Gas chromatography (GC) results are normally subject to an error of up to ±5 % of peak area. Absolute and relative values for each detected fatty acid were calculated from the areas of chromatogram peaks.

GC-mass spectrometric (GC-MS) analyses were performed on a Finnigan Trace GC-MS ultra Quadrupole GC-MS (ThermoQuest Trace DSQ, Thermo Electron Corporation, TX, USA). Data was processed with ThermoQuest Xcalibur software (Thermo Electron Corporation, TX, USA). The GC fitted with an on-column injector and a capillary HP-5 Ultra column (50 m x 0.32 mm i.d., 0.17 μm film thickness, Agilent technologies, USA) of similar polarity to that described above. Individual components were identified using mass spectral data and by comparing retention time and MS data with those obtained for authentic and laboratory standards. A full procedural blank analysis was performed concurrent to the sample batch.

### 3.3.5. Proteomics

#### 3.3.5.1. Protein extraction

Four whole fish larvae, and livers and dorsal white muscles from four fish from each tank (12 treatment$^{-1}$), were individually homogenized in Eppendorf tubes containing buffer and protease inhibitor cocktail (Roche) using Tissue-Tearor homogenator (Biospec Products, OK, USA). Proteins from whole fish and white muscle were extracted by sequential extraction as previously described (Nuez-Ortin et al., 2016); the soluble fraction in non-denaturing buffer (150 mM NaCl, 50 mM Tris at pH 8) followed by extraction of the salt-insoluble fraction in denaturing buffer (7M urea, 2M thiourea, 50 mM Tris at pH 8). Liver tissue is structurally less complex than whole fish larvae and muscle tissue. Thus, and based on the idea that the potential benefits of reduced sample complexity, if any, would not compensate for the associated increased cost of analysis, liver proteins were directly extracted in denaturing buffer. Each extraction was performed for 24h at 4°C with overnight rotation. Protein extracts were transferred into Eppendorf tubes and precipitated with 100% ethanol (9:1: v/v). Protein pellets were washed twice in 70% ethanol and re-suspended in denaturing buffer. Protein concentrations

were estimated with Bradford Protein Assay (Bio-Rad) using plate reader (Synergy TMHT, BioTek, QL, Australia). Sampling pooling was used to reduce the effect of inter-individual variability relative to the biochemical differences between fish groups exposed to different conditions (Melis et al., 2014). Protein extracts were pooled by tank (n = 4) and the volumes adjusted with denaturing buffer to achieve a concentration of 1 μg $\mu L^{-1}$ for each sample pool.

#### 3.3.5.2. Nano-liquid chromatography and tandem mass spectrometry (LTQ-Orbitrap XL)

Protein samples were trypsin-digested using standard procedures (Wilson et al., 2016) and analyzed by nano LC-MS/MS using an LTQ-Orbitrap XL and Ultimate 3000 RSLC nano HPLC system (ThermoFisher Scientific, MA, USA). Tryptic peptides (~1 μg) were loaded onto a 20 mm x 75 μm PepMap 100 trapping column (3 μm $C_{18}$) at 5 μl/min, using 98% water, 2% acetonitrile and 0.05% TFA. Peptides were separated at 0.3 μl/min on a 250 mm x 75 μm PepMap 100 RSLC column (2 μm $C_{18}$) held at 40ºC, using a stepped gradient from 97% mobile phase A (0.1% formic acid in water) to 50% mobile phase B (0.08% formic acid in 80% acetonitrile and 20 % water) comprising 3-10% B over 10 min, 10-40% B over 120 min, 40-50% B over 10 min, holding at 95% B for 10 min then re-equilibration in 3% B for 15 min. The LTQ-Orbitrap XL was controlled using Xcalibur 2.1 software in data-dependent mode and MS/MS spectra were acquired as described (Wilson et al., 2016).

#### 3.3.5.3. Database searching and criteria for protein identification

RAW files from the LTQ-Orbitrap were imported into MaxQuant software version 1.5.1.2 for peptide matching to MS/MS spectra and label-free protein quantification on the basis of median peptide intensity (LFQ) values (Cox et al., 2014). Sequential extracts of whole fish and muscle were defined as fractions of the same sample (Nuez-Ortin et al., 2016). MS/MS spectra were searched against the Salmonidae database (http://uniprot.org/taxonomy/8030; 17,795 entries) using the Andromeda search engine. Default settings for protein identification were used, including a maximum of two missed cleavages, mass error tolerances of 20 ppm then 4.5 ppm for initial and main peptide searches, respectively, 0.5 Da tolerance for fragment ions, variable oxidation of methionine and fixed carbamidomethylation of cysteine. The false discovery rates (FDR) for peptide-spectrum matches and protein identification were both set to 0.01. MaxQuant output files of the complete peptide and protein-level mass spectrometry for whole fish larvae, muscle, and liver are provided in Supplemental Table 3.1 - 3.6, respectively.

### 3.3.6. Calculations and statistical analysis

Standard formulae were used to assess growth, feed efficiency and biometrical data. Specific growth rate was calculated as SGR (% $d^{-1}$) = 100 × (ln $W_f$ / ln $W_i$) / d, where $W_f$ and $W_i$ are the final and initial weights (g) and d the number of days of the experiment. Feed efficiency ratio (g $g^{-1}$) was

determined as FER = $W_g$ / FI, where $W_g$ is weight gain (g) over the feeding trial and FI is the total feed intake (g). Fulton's condition factor was calculated as $k = W / FL^3$, where W is fish weight (g) and FL is fork length (mm). Hepato-somatic index was determined as HSI = (LW / W) × 100, where LW is liver weight (g) and W is fish weight (g).

Independent-samples t-test using SPSS v22.0 (IBM Corp., NY, USA) was used to test whether ploidy had an effect on growth indices and whole body composition. The tank effect was previously explored as a random factor within a one-way ANOVA analysis and found to be not significant. Data were checked with Levene's test to ensure normality and homogeneity of variance. Results are expressed as mean ± standard error (SEM) (n = 3), with differences between ploidy states statistically significant at p-value < 0.05.

For statistical analysis of mass spectrometry data, the "ProteinGroups" output files generated by MaxQuant analysis of whole fish, liver and muscle extracts were analysed in R (R Core Team, 2015) using the Limma package (Ritchie et al., 2015). Proteins identified on the basis of a single matching peptide were excluded. The effect of ploidy was investigated by fitting a linear model with $\log_2$ protein group intensity as the response and ploidy state as explanatory variable. An additional random effect term was also included in the model to account for possible batch effects due to the tank system used. Prior to model fitting, intensity values were normalized using cyclic loess normalization (Bolstad et al., 2003) and the method of empirical array quality weights (Ritchie et al., 2006) was used to calculate sample reproducibility and down-weight less reproducible samples. After initial model fitting, empirical Bayes (Smyth, 2004) was used to calculate moderated test statistics and Benjamini-Hochberg correction was applied to adjust p-values for multiple testing. After excluding proteins entirely that had fewer than two data points per treatment group, missing values for remaining proteins were excluded from the analysis with degrees of freedom adjusted accordingly. DAVID Bioinformatics was used to explore the gene ontology (GO) terms or functional categories representing in each sample type (Huang et al., 2009). In parallel, GOrilla was used to investigate whether ploidy had a significant pattern across multiple proteins (Eden et al., 2009). GOrilla uses a flexible threshold approach and identifies GO terms that are enriched at the top of a ranked gene list without requiring a background set. Salmonidae genes were first mapped to human orthologues using PANTHER (Mi et al., 2013), ranked by the p-value resulting from the statistical comparison of ploidy states, and then submitted to DAVID and GOrilla for functional analysis.

## 3.4. RESULTS

### 3.4.1. Growth performance, biometry, feed utilization and mortality

Over the duration of the growth trial fish had an almost 60 fold increase in body weight (Table 3.1). At 34 dph, triploids showed lower weight ($p = 0.009$, $t = 4.755$), while fork length and condition

factor (k) did not differ between ploidy states. At 109 dph, triploids showed lower weight ($p = 0.016$, $t = 4.044$) and hepato-somatic index (HSI) ($p = 0.008$, $t = 4.968$), while fork length and k were not different between ploidy states. At 162 dph, fish weight and biometric indices were not different between ploidy states. Feed intake and feed efficiency ratio (FER) were not different between ploidy states throughout the experiment. Survival did not differ between ploidy states and was overall over 90 % at the end of the experiment.

### 3.4.2. Whole body chemical composition

Ploidy did not affect the whole body dry matter, crude protein, total lipid, ash and gross energy contents at 34 dph and 109 dph (Table 3.2). However, triploids showed higher ($p = 0.037$, $t = 3.065$) whole body lipid content at 162 dph. Dry matter and lipid content increased with age, while the opposite trend was observed with crude protein and ash

### 3.4.3. Whole body lipid class and fatty acid composition

The whole body lipid class profile was generally broadly similar and was largely not affected by ploidy at 34 dph, with diploids only being significantly higher in the minor wax ester component (Table 3.3). The ploidy effect was not significant at 109 dph and 162 dph for any lipid class. The lipid class profile was predominantly composed of tryacylglycerols, which increased with age at the expense of phospholipids.

Ploidy did not affect the whole body fatty acid composition, with the exception of a minor significant ($p = 0.024$, $t = 3.562$) difference in terms of 16:0. At 34 dph and 109 dph, the predominant fatty acid group was polyunsaturated fatty acids (PUFA), and this decreased with age at the expense of monounsaturated fatty acids (MUFA), which became predominant at 162 dph. These patterns in lipid and fatty acid classes and individual fatty acids reflected the composition of feeds. Detailed chemical, lipid class and fatty acid composition of commercial feeds is provided in Appendix Table 3.1.

**Table 3.1. Growth, biometry, feed utilization and mortality of freshwater Atlantic salmon as affected by ploidy state**

| | 34 dph (late alevin) | | 109 dph (fry) | | 162 dph (parr) | |
|---|---|---|---|---|---|---|
| | Diploid | Triploid | Diploid | Triploid | Diploid | Triploid |
| Weight (g) | 0.16 ± 0.002 a | 0.14 ± 0.002 b | 1.8 ± 0.03 a | 1.6 ± 0.02 b | 8.4 ± 0.21 | 8.2 ± 0.24 |
| Fork length (mm) | 25.7 ± 0.22 | 24.9 ± 0.43 | 55.4 ± 0.63 | 54.3 ± 0.22 | 87.1 ± 0.64 | 86.9 ± 1.15 |
| $k^2$ | 0.9 ± 0.02 | 0.9 ± 0.05 | 1.0 ± 0.03 | 1.0 ± 0.02 | 1.2 ± 0.03 | 1.2 ± 0.02 |
| SGR[3] (% $d^{-1}$) | | | 3.2 ± 0.02 | 3.2 ± 0.01 | 3.1 ± 0.03 | 3.2 ± 0.03 |
| HIS[4] (%) | | | 1.7 ± 0.03 a | 1.3 ± 0.07 b | 2.1 ± 0.15 | 2.0 ± 0.16 |
| Feed intake[5] (g) | | | 1.1 ± 0.05 | 1.1 ± 0.04 | 7.1 ± 0.26 | 7.1 ± 0.16 |
| FER[6] | | | 1.5 ± 0.03 | 1.4 ± 0.05 | 1.1 ± 0.07 | 1.1 ± 0.02 |
| Survival (%) | | | 97.3 ± 1.90 | 97.1 ± 1.38 | 94.4 ± 2.63 | 91.3 ± 3.95 |

Data expressed as mean ± SEM (n = 3). Different superscripts within a row and sampling time denote significant differences ($p < 0.05$)

[1]Days post-hatching

[2]Fulton's condition factor

[3]Specific growth rate

[4]Hepato somatic index

[5]Intake expressed as wet weight

[6]Feed efficiency ratio

**Table 3.2. Carcass chemical composition (% DM) of freshwater Atlantic salmon as affected by ploidy**

| | 34 dph[1] (late alevin) | | 109 dph (fry) | | 162 dph (parr) | |
|---|---|---|---|---|---|---|
| | Diploid | Triploid | Diploid | Triploid | Diploid | Triploid |
| Dry matter (% WW) | 22.0 ± 0.05 | 23.5 ± 0.61 | 24.1 ± 0.06 | 23.7 ± 0.23 | 27.3 ± 0.14 | 26.3 ± 0.23 |
| Crude protein | 68.8 ± 0.34 | 68.2 ± 0.41 | 61.9 ± 0.32 | 61.3 ± 0.25 | 56.8 ± 1.48 | 55.5 ± 0.49 |
| Total lipid | 22.7 ± 1.31 | 23.0 ± 0.95 | 22.7 ± 0.73 | 24.3 ± 0.64 | 27.2 ± 0.58 b | 29.2 ± 0.33 a |
| Ash | 8.7 ± 0.36 | 8.4 ± 0.18 | 8.5 ± 0.17 | 8.8 ± 0.14 | 7.6 ± 0.12 | 7.6 ± 0.04 |
| Gross energy (kJ/g DM) | 26.6 ± 0.13 | 26.6 ± 0.20 | 25.4 ± 0.17 | 24.7 ± 0.32 | 26.9 ± 0.13 | 27.1 ± 0.21 |

[1]Days post-hatching

Data expressed as mean ± SEM (n=3). Different superscripts within row and sampling time denotes a significant difference between ploidy states ($p<0.05$)

**Table 3.3. Total fatty acid content (mg $g^{-1}$ lipid), lipid classes (% total lipid) and fatty acid composition (% of total fatty acid) of carcasses of freshwater Atlantic salmon as affected by ploidy state**

| | 34 dph[1] (late alevin) | | 109 dph (fry) | | 162 dph (parr) | |
|---|---|---|---|---|---|---|
| | Diploid | Triploid | Diploid | Triploid | Diploid | Triploid |
| *Total fatty acid* | 705.7 ± 90.00 | 703.4 ± 73.09 | 873.7 ± 24.18 | 863.1 ± 26.69 | 913.5 ± 30.52 | 889.7 ± 28.07 |
| *Lipid class* | | | | | | |
| Wax esters | 3.8 ± 0.26 a | 2.8 ± 0.15 b | 1.7 ± 0.22 | 1.4 ± 0.43 | 1.3 ± 0.28 | 1.3 ± 0.12 |
| Triacylglycerols | 47.8 ± 3.31 | 46.4 ± 3.95 | 77.8 ± 1.52 | 77.9 ± 2.74 | 86.3 ± 0.50 | 85.9 ± 1.17 |
| Free fatty acids | 1.6 ± 0.52 | 0.9 ± 0.12 | 1.1 ± 0.20 | 1.3 ± 0.35 | 1.2 ± 0.11 | 1.1 ± 0.11 |
| Sterols | 4.9 ± 0.62 | 4.3 ± 0.17 | 2.8 ± 0.51 | 2.3 ± 0.30 | 2.1 ± 0.35 | 1.5 ± 0.05 |
| Phospholipids | 41.9 ± 4.51 | 45.5 ± 4.15 | 16.6 ± 0.65 | 17.2 ± 2.48 | 9.0 ± 0.77 | 10.2 ± 1.23 |
| *Fatty acid composition* | | | | | | |
| 14:0 | 1.7 ± 0.10 | 2.0 ± 0.18 | 4.6 ± 0.24 | 4.6 ± 0.24 | 4.1 ± 0.10 | 4.3 ± 0.25 |
| 16:0 | 13.4 ± 0.21 | 13.7 ± 0.39 | 16.7 ± 0.05 b | 16.9 ± 0.05 a | 14.2 ± 0.14 | 14.1 ± 0.59 |
| 17:0 | 0.3 ± 0.01 | 0.3 ± 0.01 | 0.4 ± 0.04 | 0.4 ± 0.01 | 0.3 ± 0.00 | 0.3 ± 0.04 |
| 18:0 | 7.0 ± 0.19 | 7.1 ± 0.40 | 4.0 ± 0.03 | 4.0 ± 0.03 | 3.1 ± 0.02 | 3.1 ± 0.19 |
| Other SFA[2] | 0.3 ± 0.03 | 0.4 ± 0.01 | 0.9 ± 0.03 | 0.8 ± 0.03 | 0.9 ± 0.02 | 0.8 ± 0.02 |
| Total SFA | 22.8 ± 0.39 | 23.5 ± 0.54 | 26.5 ± 0.22 | 26.8 ± 0.26 | 22.6 ± 0.19 | 22.6 ± 0.94 |
| 16:1n-7 | 5.7 ± 0.07 | 5.9 ± 0.09 | 7.4 ± 0.03 | 7.5 ± 0.06 | 5.2 ± 0.06 | 5.1 ± 0.21 |
| 18:1n-7 | 4.5 ± 0.05 | 4.5 ± 0.04 | 3.7 ± 0.04 | 3.7 ± 0.02 | 3.3 ± 0.02 | 3.2 ± 0.09 |
| 18:1n-9 | 16.0 ± 0.23 | 15.9 ± 0.28 | 13.5 ± 0.47 | 13.1 ± 0.06 | 22.9 ± 0.18 | 25.0 ± 2.90 |
| 20:1n-7 | 0.2 ± 0.03 | 0.2 ± 0.02 | 0.3 ± 0.02 | 0.3 ± 0.02 | 0.3 ± 0.01 | 0.3 ± 0.02 |
| 20:1n-9 | 0.6 ± 0.04 | 0.6 ± 0.02 | 1.4 ± 0.14 | 1.1 ± 0.01 | 4.7 ± 0.12 | 4.4 ± 0.14 |
| 22:1n-11 | 0.1 ± 0.03 | 0.0 ± 0.02 | 0.8 ± 0.18 | 0.7 ± 0.03 | 4.6 ± 0.21 | 4.3 ± 0.29 |
| 24:1n-9 | 0.3 ± 0.01 | 0.3 ± 0.03 | 0.6 ± 0.00 | 0.6 ± 0.14 | 0.7 ± 0.03 | 0.7 ± 0.05 |
| Other MUFA[3] | 1.3 ± 0.04 | 1.3 ± 0.17 | 2.0 ± 0.10 | 1.7 ± 0.04 | 2.8 ± 0.04 | 2.8 ± 0.20 |
| Total MUFA | 28.7 ± 0.35 | 28.8 ± 0.20 | 29.6 ± 0.68 | 28.6 ± 0.04 | 44.6 ± 0.52 | 45.7 ± 1.72 |
| 18:2n-6 | 4.4 ± 0.08 | 4.4 ± 0.14 | 7.6 ± 0.09 | 7.7 ± 0.05 | 7.5 ± 0.05 | 7.2 ± 0.19 |
| 20:4n-6 | 2.0 ± 0.04 | 2.0 ± 0.02 | 0.8 ± 0.02 | 0.8 ± 0.02 | 0.5 ± 0.01 | 0.5 ± 0.02 |
| Other n-6[4] | 1.5 ± 0.03 | 1.4 ± 0.02 | 1.6 ± 0.20 | 1.5 ± 0.01 | 1.3 ± 0.01 | 1.4 ± 0.11 |
| Total n-6 | 7.9 ± 0.07 | 7.9 ± 0.11 | 10.0 ± 0.11 | 9.9 ± 0.07 | 9.3 ± 0.05 | 9.0 ± 0.25 |
| 18:3n-3 | 0.9 ± 0.02 | 0.9 ± 0.03 | 1.3 ± 0.05 | 1.2 ± 0.01 | 2.2 ± 0.02 | 2.2 ± 0.06 |
| 18:4n-3 | 1.3 ± 0.02 | 1.4 ± 0.05 | 2.0 ± 0.02 | 2.0 ± 0.01 | 1.7 ± 0.03 | 1.6 ± 0.06 |
| 20:4n-3 | 1.6 ± 0.02 | 1.6 ± 0.05 | 1.0 ± 0.12 | 0.9 ± 0.00 | 0.8 ± 0.01 | 0.8 ± 0.07 |
| 20:5n-3 | 11.9 ± 0.07 | 11.8 ± 0.05 | 6.8 ± 0.16 | 7.2 ± 0.12 | 3.2 ± 0.07 | 3.2 ± 0.11 |
| 22:5n-3 | 6.1 ± 0.06 | 5.9 ± 0.09 | 2.7 ± 0.05 | 2.8 ± 0.03 | 1.4 ± 0.01 | 1.4 ± 0.02 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 22:6n-3 | 17.1 ± 0.22 | 16.5 ± 0.05 | 15.7 ± 0.29 | 16.4 ± 0.18 | 11.7 ± 0.21 | 11.0 ± 0.25 |
| Other n-3[5] | 0.7 ± 0.02 | 0.7 ± 0.03 | 1.8 ± 0.07 | 1.7 ± 0.06 | 1.2 ± 0.02 | 1.1 ± 0.05 |
| Total n-3 | 39.6 ± 0.25 | 38.8 ± 0.26 | 31.1 ± 0.55 | 32.2 ± 0.26 | 22.2 ± 0.34 | 21.3 ± 0.47 |
| Other PUFA | 0.3 ± 0.01 | 0.3 ± 0.00 | 0.4 ± 0.12 | 0.2 ± 0.00 | 0.2 ± 0.00 | 0.2 ± 0.07 |
| Total PUFA | 47.8 ± 0.22 | 47.0 ± 0.37 | 41.5 ± 0.64 | 42.3 ± 0.29 | 31.6 ± 0.37 | 30.5 ± 0.75 |
| n-3:n-6[6] | 5.0 ± 0.07 | 4.9 ± 0.04 | 3.1 ± 0.05 | 3.2 ± 0.03 | 2.4 ± 0.03 | 2.4 ± 0.02 |
| DHA:EPA[7] | 1.5 ± 0.02 | 1.4 ± 0.01 | 2.3 ± 0.02 | 2.3 ± 0.03 | 3.6 ± 0.02 | 3.4 ± 0.08 |

Data expressed as mean ± SEM (n = 3). Different superscripts within row and sampling time denotes a significant difference between ploidy states ($p<0.05$).

[1]Days post-hatching

[2]Includes 15:0, 20:0, 21:0, 22:0, 23:0 and 24:0

[3]Includes 16:1n-5, 16:1n-9, 18:1n-5, 20:1n-7, 22:1n-9 and 24:1n-9

[4]Includes 20:2n-6, 22:5n-6, and 22:4n-6

[5]Includes 21:5n-3, 24:6n-3 and 24:5n3

[6]n-3:n-6 ratio

[7]DHA:EPA ratio

### 3.4.4. Proteomics

#### 3.4.4.1. Overview

A total of 614 proteins that met filter criteria for protein quantitation were identified from whole fish alevin (Supplemental Table 3.7). At 109 dph and 162 dph, 478 and 460 proteins were quantifiable in white muscle tissue, respectively (Supplemental Tables 3.8 and 3.9), while 756 and 737 proteins were quantifiable in liver tissue at 109 dph and 162 dph, respectively (Supplemental Tables 3.10 and 3.11). Figure 3.2 shows significantly represented (adjusted p-value<0.05) biological process terms across sample types as determined by DAVID analysis. Biological processes related to protein synthesis (e.g. translational elongation, ribosome biogenesis and assembly) and degradation (e.g. regulation of cellular protein metabolic process), and to energy metabolism (e.g. generation of precursor metabolites and energy, oxidative phosphorylation, carbohydrate catabolic process), were among the most represented across all sample types. Lipid metabolism-related processes showed significant representation in liver (e.g. fatty acid beta-oxidation, fatty acid metabolic process) and white muscle (e.g. lipid transport) tissues.

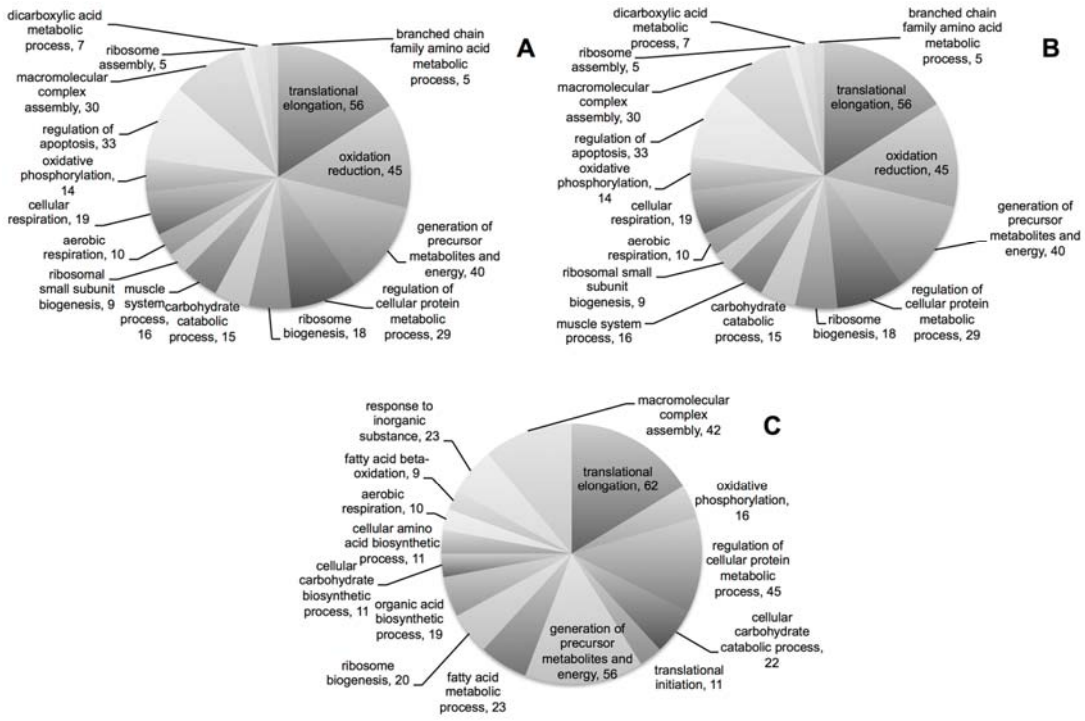


**Figure 3.2. Pie charts showing representative gene (GO) biological process terms, and the number of associated proteins, in whole fish alevin (A), white muscle (B) and liver (C) tissues as determined by DAVID Bioinformatics**. Terms are representative of annotation clusters and only those with an adjusted p-value of <0.05 are summarized.

#### 3.4.4.2. Whole fish alevin (34 dph)

Principal component analysis (PCA) showed separation between ploidy states (Figure 3.3A), but statistical comparison of intensity values did not identify significant (based on adjusted p-value < 0.05) differences in protein abundance for any specific protein. As shown by the volcano plot (Figure 3.3B), fold changes in protein abundance between ploidy states were generally below 2 ($log_2$ fold change < 1). GO ontology analysis using GOrilla showed a significant (adjusted p-value < 0.05) pattern across multiple proteins and the corresponding molecular function terms. These included "NADH dehydrogenase" (11 proteins), "hydrogen ion transmembrane transporter" (9 proteins) and "structural constituent of the ribosome" (38 proteins).

#### 3.4.4.3. Muscle tissue (109 dph and 162 dph)

PCA showed maximum separation between sampling times but considerable overlap between ploidy states (Figure 3.3C), and no resulting significant (based on adjusted p-value < 0.05) differences in protein abundance between ploidy states were observed. The volcano plot is a combination of two datasets and represents fold changes between ploidies and between sampling times (Figure 3.3D). Fold changes between proteins as affected by ploidy were generally below 2 ($log_2$ fold change < 1) at both 109 dph and 162 dph . Comparison between sampling times validated the capability of our proteomics approach to detect statistical significance in muscle tissue between different physiological states, with 63 proteins showing significant fold changes ranging from 2 to 208 ($log_2$ fold change 1.2 - 7.7). No significant GO terms across multiple genes were identified as affected by ploidy.

#### 3.4.4.4. Liver tissue (109 dph and 162 dph)

As found for the muscle samples, PCA showed considerable overlap between ploidy states (Figure 3.3E) and fold changes between proteins as affected by ploidy were generally very low at both 109 dph and 162 dph (Figure 3.3F). Statistical comparison of intensity values did not identify significant differences between ploidy states, whereas 16 proteins were significantly altered between sampling times, with fold changes ranging from 2 to 45.3 ($log_2$ fold change 1 – 5.5). No significant GO terms across multiple genes were identified as affected by ploidy.

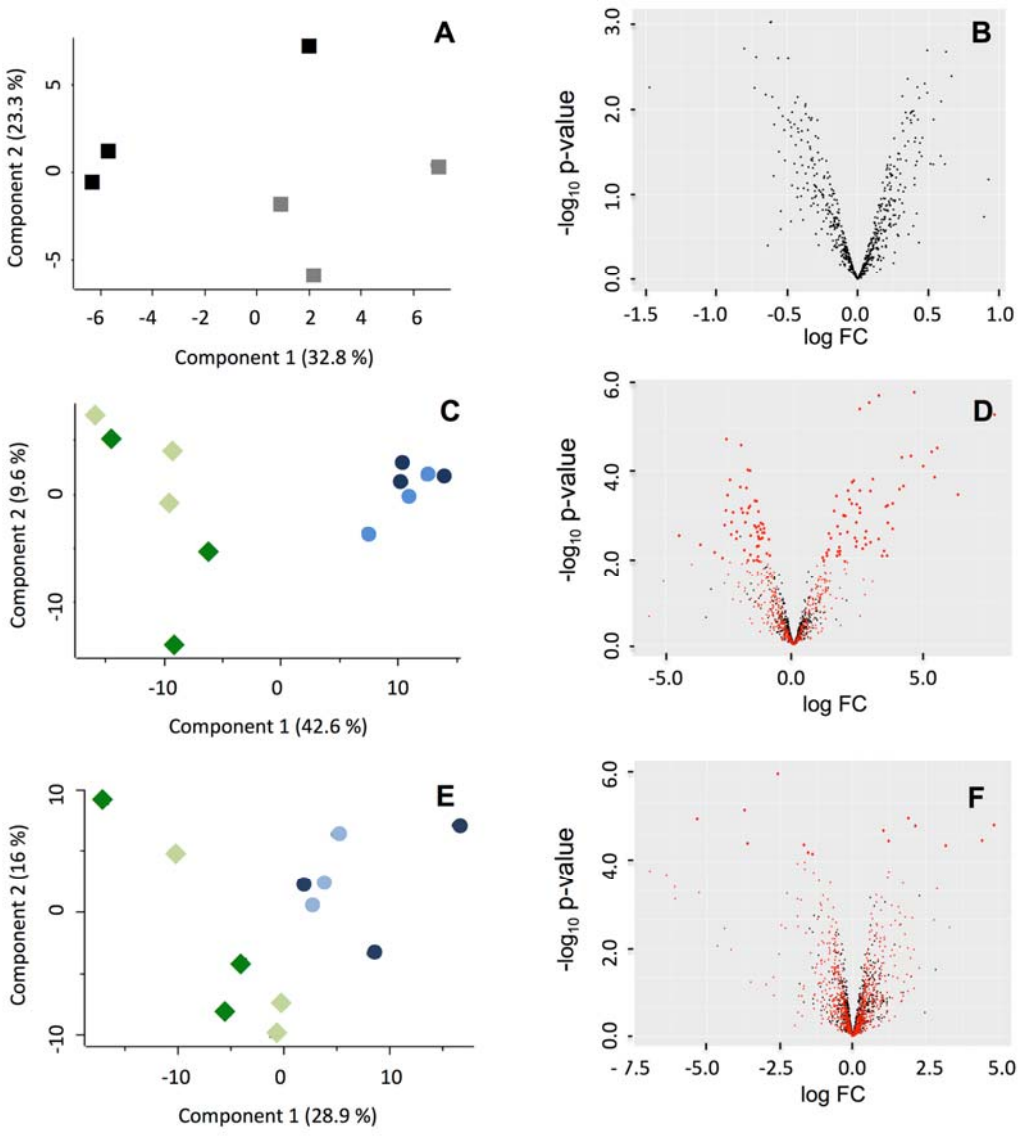


**Figure 3.3. Principal component analysis (PCA) and volcano plots of the whole fish larvae (A, B), white muscle (C, D) and liver (E, F) proteome profiles as affected by ploidy.** In A, grey and black represent diploid and triploid whole fish, respectively, at 34 dph. Data points in C and E are plotted in different colors based on sampling time; green and blue represent 109 and 162 dph, respectively, as affected by ploidy (light: diploids; dark: triploids). Volcano plots show log fold changes between ploidy states (black) and between sampling times (red). Thicker red circles in white muscle (D) and liver (F) plots represent proteins with significant differential expression between sampling times.

## 3.5. DISCUSSION

This is the first study to use shotgun proteomics to examine triploid physiology of freshwater Atlantic salmon under optimal growing conditions. Furthermore, we used multi-tissue and time-series sampling, combined with measurements of growth and body composition, to achieve an integrated exploration of triploid-specific physiological and phenotypic traits. The main finding of the present study was that ploidy resulted only in subtle differences in growth performance and whole body chemical composition, and the same pattern was reflected at the proteome level.

### 3.5.1. Growth performance and survival

Knowledge of early life history is important because it can dictate later performance (Johnston et al., 2003, Macqueen et al., 2008). Previous recommendations in relation to egg quality (Taylor et al., 2011), incubation temperature (Fraser et al., 2013, Fraser et al., 2014) and dietary phosphorus supply (Fjelldal et al., 2016) towards optimizing triploid survival, growth and development, were followed in the present study. The observed growth pattern, with lower triploid weight prior to first feeding (34 dph) and during the fry interval (34 dph - 109 dph) but reaching comparable weight at the parr stage (162 dph), concurs with previous studies comparing diploid and triploid salmon in freshwater under optimal growing conditions and reared separately (Carter et al., 1994, McGeachy et al., 1995, O'Flynn et al., 1997, Cotter et al., 2002, Taylor et al., 2011, Taylor et al., 2013, Amoroso et al., 2016a). Although triploids are generally smaller during the larval stage up to several weeks or months after first feeding, they eventually catch up with diploids once feeding is established and even reach superior growth that is carried over through smolting. Triploid fry (109 dph) showed lower HSI, suggesting either less metabolically active liver or delayed somatic development, which might have contributed to the lower triploid weight observed prior to first feeding and during the fry interval (34 dph - 109 dph). This result is consistent with observations by Cotter et al (2002), who reported lower visceral weight in freshwater triploid salmon of similar size, though this difference was not statistically confirmed. Differences in whole fish and visceral weight following first feeding can be attributed to the higher incidence of non-feeders in the triploid group (Carter et al., 1994, Cotter et al., 2002) and to differences in fish distribution in the water column and tank, and consequent access to feed (McGeachy et al., 1995). In the present study, it was observed that triploids were approximately one week slower than diploids to disperse though the water column and access the surface for first feeding, showing a tendency to feed off the bottom of the crate with frequent rejection of feed pellets (Amoroso et al., 2016a). Despite delayed triploid first feeding, triploids caught up diploid intake over time and were equally efficient at converting feed into growth. This is supported by previous studies of juvenile salmonids, including Atlantic salmon, demonstrating that nutrient digestibility and feed utilization is not affected by ploidy (Oliva‐Teles and Kaushik, 1990, Burke et al., 2010, Tibbetts et al., 2013, Sacobie et al., 2015).

Survival was not affected by ploidy, and this consistent with most studies examining the period between first feeding and seawater transfer of salmon (McGeachy et al., 1995, O'Flynn et al., 1997, Burke et al., 2010, Taylor et al., 2011, Tibbetts et al., 2013). Assuming optimal egg quality and growing conditions, triploid mortality during exogenous feeding has been associated with the use of regular diploid diets that do not meet triploid phosphorus requirements (Fjelldal et al., 2016). Higher triploid mortality is however more common at the end of incubation period (Amoroso et al., 2016a) and this likely relates to undesirable but unknown effects caused by the pressure shock (Piferrer et al., 2009).

### 3.5.2. Whole body composition

Salmon is an important contributor of high quality protein and omega-3 long-chain ($\geq C_{20}$) polyunsaturated fatty acids (n-3 LC-PUFA) to the human diet, thus it is important to understand whether ploidy *per se*, rather than the effect of factors associated with the suppression of gonadogenesis, affects whole body chemical composition. Early freshwater stages offer a physiological window to avoid maturation-related signals affecting nutrient repartitioning, particularly of lipids and fatty acids. The observed higher whole body lipid content in triploid parr (162 dph) is consistent with previous studies in freshwater Atlantic salmon (Burke et al., 2010, Tibbetts et al., 2013) and in juveniles of other salmonid species (Oliva‐Teles and Kaushik, 1990, Sacobie et al., 2015) that also confirmed that this effect is dependent on fish size. As previously observed (Segato et al., 2006), this effect became significant after more than 12 weeks on formulated feeds, and should be predominant in the fillet composition of fish reaching market size (Poontawee et al., 2007, Skare, 2015). Higher lipid accumulation in triploid fish has been attributed to larger cells and the consequent increased lipid storage capacity (Johnston et al., 1999, Nanton et al., 2007). Also supporting previous evidence provided by the aforementioned studies in juvenile salmonids, the whole body dry matter, protein, ash and energy content was not ploidy-dependent throughout the experiment, and this same pattern has been observed in the fillet composition of market-sized salmonids (Bjørnevik et al., 2004, Cleveland et al., 2012, Skare, 2015).

Lipid classes and fatty acids contribute to cell structure and provide stored fuel, thus their utilization is important during the rapid growth in early life stages of fish (Hamre et al., 2013). The present study is the first examination of the ploidy effect on the whole body composition of lipid classes and fatty acids in Atlantic salmon during early freshwater stages comprising endogenous and exogenous feeding. Prior to first feeding (34 dph), the minor wax ester fraction was lower in triploid fish. Given the observed lower triploid weight at this sampling time, and the dynamic rate of change in the lipid class proportions during endogenous feeding (i.e. changes observed every four days) (Tocher et al., 1985), the difference in the wax ester fraction is possibly a consequence of a slight delay in triploid development. The lack of any ploidy effect on individual fatty acids during endogenous feeding

indicates that the synthesis and retention of n-3 LC-PUFA or other essential fatty acids is not ploidy-dependent during this period.

During exogenous feeding, the whole body lipid class and fatty acid composition was generally similar to those of feeds, though a few exceptions were found. Wax esters and free fatty acids were higher in feed than in fish, which can be explained by the slow hydrolysis and subsequently low bioavailability of the former (Bogevik, 2011), and by the utilization of the latter as aerobic fuel substrate (Hepher, 1988). EPA was higher in feed than in fish, while DHA was higher in fish than in the feed, this being the result of the preferential β-oxidation of EPA over DHA and consequently the greater incorporation of DHA into lipid membranes (Codabaccus et al., 2012). Whole body lipid class and fatty acid composition was not affected by ploidy, with the exception of a minor difference in 16:0 at the fry stage (109 dph). There exists a relationship between phospholipid and n-3 LC-PUFA availability, and productive parameters such as performance, survival, and deformity prevalence, during the early stages of exogenous feeding (Gisbert et al., 2005, Hamre et al., 2013). The present results indicate that, when these nutrients are supplied in sufficient and balanced amounts, the effect of ploidy on productive differences should not be attributed to differential utilization of these nutrients. Only one study has previously evaluated the effect of ploidy on the fatty acid composition of juvenile fish (Ozório et al., 2012). This was conducted in fish supplemented with L-carnitine and showed less accumulation of saturated fatty acids (SFA) in muscle and liver tissues of triploids. Although this difference was linked to a possible effect of ploidy on the selectivity of fatty acid oxidation or *de novo* synthesis, it could rather be ascribed to the significantly higher feed intake by diploids, or even to differential L-carnitine utilization rather than to the ploidy effect *per se* on fatty deposition. More attention has been devoted to the ploidy effect on the muscle fatty acid composition of maturing fish, particularly salmonids. Triploid females of rainbow trout tended to accumulate more SFA and MUFA and contained lower PUFA, mostly n-6 PUFA, relative to diploids (Manor et al., 2012, Ribeiro et al., 2012, Salem et al., 2013, Manor et al., 2014). A mixed sex population of Atlantic salmon showed higher MUFA and n-3 LC-PUFA contents but no differences in SFA or n-6 PUFA (Taylor et al., 2013). Differences among studies clearly demonstrate that the degree of variability in body composition between ploidy states is caused, in addition to factors associated to the suppression of gonadogenesis, by other endogenous and exogenous factors such as sex, age, species, and dietary fatty acid composition. These variables were excluded in the present study, with the results leading to the conclusion that the whole body fatty acid composition of salmon during early freshwater stages is largely not affected by ploidy.

### 3.5.3. Proteomics

Exploratory proteomic profiling of whole fish larvae or fish tissues has been used to understand dietary-, environment- or developmental- induced physiological changes (Rodrigues et al., 2012). The present study is the first attempt to use such an approach to gain insight into the physiological consequences of triploidization in freshwater Atlantic salmon. While our quantitative comparison

among whole fish larvae, muscle and liver proteomes identified statistically significant protein differences related to sampling times, no differentially abundant proteins were identified between ploidy states. While all such analyses are a balance between statistical power and effect size, these results demonstrate that changes due to ploidy are considerably less dramatic than changes due to fish growth.

In whole fish alevin (34 dph), and despite the lack of a significant effect of ploidy on the expression of individual proteins, GO analysis detected a significant pattern across multiple proteins. The functional terms "NADH dehydrogenase" and "hydrogen ion transmembrane activity" were affected by ploidy, with 18 out of 19 proteins comprised in these terms increased abundance between 10-50% in triploids relative to diploids. These are mitochondrial processes involved in the generation of ATP via oxidative phosphorylation, the efficiency of which is vital for nutrient utilization and proper development (Eya et al., 2014, Bermejo-Nogales et al., 2015, Eya et al., 2015). Since changes in oxidative phosphorylation parallel changes in nutrient utilization and development, a slight increase in energy demand and in the corresponding mitochondrial ATP production may link with the proposed delay in triploid development, as also supported by the lower values in triploid weight and whole body wax ester content. Alternatively, increased mitochondrial activity has been associated with increased stress (Liesa and Shirihai, 2013), which has been in turn attributed to triploidization (Piferrer et al., 2009). The fact that significantly enriched GO terms were observed at alevin stage, but not at fry and parr stages where the effects on weight were also minimal, raises the possibility that triploidization-related stress altered mitochondrial activity in the early stages but this effect was attenuated at later stages.

The effect of ploidy on growth regulation has been linked to the larger size but lower density of triploid cells, implying that hypertrophy is the preferential strategy for muscle growth in triploids while they have a reduced capacity for hyperplasia (Johnston et al., 1999). Proteomics has been used to investigate muscle physiological adaptation in relation to hypertrophy and hyperplasia in fish (Reddish et al., 2008, Rescan et al., 2013) and mammals (Bouley et al., 2005, Hamelin et al., 2006, Ojima et al., 2014), and it is therefore a valuable tool to explore the ploidy effect on muscle growth dynamics. Many proteins related to both hypertrophy (e.g. contractile proteins, enzymes involved in glycolysis and other metabolic processes, molecular chaperones) and hyperplasia (e.g. galectins, follistatins, semaphorins, extracellular matrix components such as collagen alpha-I) were identified in fry (109 dph) and parr (162 dph) muscle in the present study. However, no significant alterations in these proteins were identified, suggesting that, like observations in growth and whole body lipid content, the effect on muscle hypertrophy and hyperplasia was minimal. Fish used in the present study were all-female with a fork length ranging from 5 to 9 cm between 109 dph and 162 dph. Sex and fork length are two factors to consider that may have determined the lacking ploidy effect on hyperplasia-related proteins is sex. In Atlantic salmon of similar size (~ 7 cm), reduced hyperplasic growth in triploids was significantly detected in a normal-sex-ratio population but not in the relative all-female population (Johnston et al.,

1999), indicating that the ploidy effect on the hyperplasic capacity in this length range is more accentuated in males than in females.

Muscle and liver are highly metabolic tissues that play a crucial role in nutrient metabolism and growth. Regulation of protein deposition in white muscle determines growth rate (Houlihan and Laurent, 1987), while liver is a crucial site for fatty acid metabolism and body lipid homeostasis (Monroig et al., 2010). Within the limits of the proteome coverage achieved in this study, the absence of differences in protein expression in muscle and liver at the fry (109 dph) and parr (162 dph) stages suggests a null or very subtle effect of ploidy on the mechanisms regulating growth and nutrient utilization under optimal growing conditions. Cleveland and Weber (2013) observed that reduced rates of protein degradation are a mechanism contributing to faster recovery growth in triploid juveniles of rainbow trout during the re-feeding phase following feed deprivation. However, this response was not confirmed under the present conditions of optimal feeding and comparable intake between ploidy groups, and where proteins involved in the ubiquitin-proteasome (e.g. proteasome subunits, ubiquitin conjugating enzymes) and autophagic-proteolytic (e.g. cathepsins) pathways were detected (e.g. regulation of cellular protein metabolic process) in both the muscle and liver proteomes of fry (109 dph) and parr (162 dph). Higher levels of fatty acid synthesis, lower levels of $\beta$-oxidation, and differences in lipid binding and transport, were reported in liver of triploid of rainbow trout relative to diploids during the period of sexual maturation (Manor et al., 2014, Manor et al., 2015, Cleveland and Weber, 2016). In the present study, the GO terms "fatty acid $\beta$-oxidation" and "fatty acid metabolic processes", the latter comprising fatty acid binding proteins, were significantly represented in liver tissue, but none of the encompassed proteins confirmed the ploidy effect. This finding suggests that previously observed changes in lipid metabolism were hormonally driven and not caused by the effect of ploidy *per se*. Notably, a number of energy metabolism-related processes were also predominant in both muscle and liver, implying sufficient proteome coverage to potentially detect ploidy differences in energy utilization. This lack of ploidy effect at the muscle and liver proteome level was supported by the subtle differences in growth and whole body composition, and possibly preceded by optimal growing conditions that did not exploit the ploidy effect with sufficient magnitude. This response, derived from what has been described as the dosage compensation mechanism (Birchler et al., 2001), indicates that despite the extra set of chromosomes in the triploid and the possible differences in cell size, the cytoplasmic:nuclear ratio as well as concentration of regulatory factors are maintained and allow for normal physiological development of these tissues. The dosage mechanism, however, can be unbalanced by a situation involving increased gene regulation. As observed in fish, similar levels of transcription between diploids and triploids occurred during optimal conditions of growth (Shrimpton et al., 2007, Shrimpton et al., 2012), while the ploidy effect on transcription levels was detected in sub-optimal situations such as feed deprivation (Cleveland and Weber, 2013) or disease challenge (Ching et al., 2010). Based on the present results, we speculate that the dosage compensation mechanism was

maintained at the muscle and liver proteome level in the Atlantic salmon fry and parr stages, this being attributed to the lack of stress or non-optimal growing conditions required to alter the levels of protein expression in response to ploidy.

### 3.5.4. Conclusion

The early freshwater stages (alevin, fry and parr) of Atlantic salmon provide the opportunity to assess the ploidy effect *per se* on performance and body composition and to explore the underlying physiological mechanisms in the absence of maturation-related signals associated with suppressed gonad development. Triploid salmon developed subtle differences in weight and whole body lipid composition in relation to the diploid counterpart. The lack of detectable differences in individual protein expression was consistent with these data, suggesting that higher levels of stress such as sub-optimal growth conditions are required before ploidy effects are manifested. Given this is the first study to investigate the proteome response of early freshwater Atlantic salmon, the improved understanding that all-female triploids and diploids had a similar physiological response suggests that optimal husbandry conditions are likely similar between ploidies. However, results of other studies from mixed sex triploid populations cannot be directly translated to the commercial situation where all-female triploids are produced. Further proteomics research applied to triploidy should aim to better understand the physiological consequences of triploidization during periods of higher triploid mortality, such as incubation and the early alevin stage, and to identify bone- and cartilage- specific molecular mechanisms involved in the development of skeletal deformities in later freshwater stages.

"The mass spectrometry proteomics data for whole fish larvae, white muscle and liver have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD005452, PXD005451 and PXD005450".

# CHAPTER 4

# Preliminary validation of a high docosahexaenoic acid (DHA) and α-linolenic acid (ALA) dietary oil blend: tissue fatty acid composition and liver proteome response in Atlantic salmon (*Salmo salar*) smolts

Published as Nuez-Ortin, W. G., Carter, C. G., Wilson. R., Cooke, I. and Nichols, P. D. 2016. Preliminary validation of a high docosahexaenoic acid (DHA) and alpha-linolenic acid (ALA) dietary oil blend: tissue fatty acid composition and liver proteome response in Atlantic salmon (*Salmo salar*) smolts. PLos One 11(8): e0161513.

## 4.1. ABSTRACT

Marine oils are important to human nutrition as the major source of docosahexaenoic acid (DHA), a key omega-3 long-chain ($\geq C_{20}$) polyunsaturated fatty acid (n-3 LC-PUFA) that is low or lacking in terrestrial plant or animal oils. The inclusion of fish oil as main source of n-3 LC-PUFA in aquafeeds is mostly limited by the increasing price and decreasing availability. Fish oil replacement with cheaper terrestrial plant and animal oils has considerably reduced the content of n-3 LC-PUFA in flesh of farmed Atlantic salmon. Novel DHA-containing oils with high α-linolenic acid (ALA) content will be available from transgenic oilseeds plants in the near future as an alternative for dietary fish oil replacement in aquafeeds. As a preliminary validation, we formulated an oil blend (TOFX) with high DHA and ALA content using tuna oil (TO) high in DHA and the flaxseed oil (FX) high in ALA, and assessed its ability to achieve fish oil-like n-3 LC-PUFA tissue composition in Atlantic salmon smolts. We applied proteomics as an exploratory approach to understand the effects of nutritional changes on the fish liver. Comparisons were made between fish fed a fish oil-based diet (FO) and a commercial-like oil blend diet (fish oil + poultry oil, FOPO) over 89 days. Growth and feed efficiency ratio were lower on the TOFX diet. Fish muscle concentration of n-3 LC-PUFA was significantly higher for TOFX than for FOPO fish, but not higher than for FO fish, while retention efficiency of n-3 LC-PUFA was promoted by TOFX relative to FO. Proteomics analysis revealed an oxidative stress response indicative of the main adaptive physiological mechanism in TOFX fish. While specific dietary fatty acid concentrations and balances and antioxidant supplementation may need further attention, the use of an oil with a high content of DHA and ALA can enhance tissue deposition of n-3 LC-PUFA in relation to a commercially used oil blend.

## 4.2. INTRODUCTION

The importance of seafood in human nutrition and the ability of aquaculture, as it meets the increasing demand for seafood, to provide nutrients traditionally supplied by seafood, particularly key fatty acids, are central issues in global food security (Watson et al., 2015). The absolute content of omega-3 long-chain ($\geq C_{20}$) polyunsaturated fatty acid (n-3 LC-PUFA), particularly docosahexaenoic acid (22:6n-3, DHA) and eicosapentaenoic acid (20:5n-3, EPA), is one of the critical contributions that Atlantic salmon makes to human diet and nutrition (Nichols et al., 2014), and the benefits of an adequate dietary supply of n-3 LC-PUFA are well documented (Delgado-Lista et al., 2012, Bell et al., 2014). Current dietary recommendations for weekly intake of n-3 LC-PUFA range between 2 and 14 g (NHMRC, 2006, EFSA, 2010), equivalent to at least two weekly servings of 100 g of fatty fish such as Atlantic salmon. However, concerns regarding the actual content of n-3 LC-PUFA in the fillet of farmed Atlantic salmon and the potential health benefits delivered to the consumer have been raised (Fry et al., 2016). As shown by recent reports from Australia and Europe, current absolute concentrations of the n-3 LC-PUFA have dropped to half (~ 1000 mg per 100 g of muscle) in the last decade and/or show high variability among retailers (Henriques et al., 2014, Nichols et al., 2014). This decrease in n-3 LC-PUFA content was attributed by the same authors to industry feeding practices, which are a consequence of reduced fish oil availability, competition from other industries and increasing price. Terrestrial vegetable oils and rendered animal fats are used to replace fish oil in salmon aquafeeds; and although less expensive and more readily available than fish oil, they are low or lacking in n-3 LC-PUFA (Emery et al., 2014, Nichols et al., 2014). Therefore, enhancing the actual nutritional value of Atlantic salmon fillet necessarily requires the inclusion in aquafeeds of new and sustainable oils with higher n-3 LC-PUFA content than those currently in use.

A parallel line of thought is that sustainable feed formulation should also aim to promote efficient tissue deposition of n-3 LC-PUFA. The foundation for this is that tissue fatty acid composition is not only dependent on dietary fatty acid composition, but also on the respective metabolic fates such as utilization for energy, bioconversion or *de novo* production (Turchini and Francis, 2009). The concept of a "n-3 LC PUFA sparing effect" was revised by Codabaccus et al. (2012), concluding that equally important to the absolute dietary supply of n-3 LC-PUFA is an increase in the DHA:EPA ratio that promotes enhanced tissue deposition efficiency. Concurrently, the efficiency of tissue deposition of n-3 LC-PUFA in salmonid teleosts can be promoted by increasing the availability of the α-linolenic acid (18:3n-3, ALA) precursor, which can be further desaturated and elongated (Turchini and Francis, 2009, Emery et al., 2013, Hixson et al., 2014b), or alternatively, preferentially oxidized over n-3 LC-PUFA if present in high amounts (Budge et al., 2011, Sanden et al., 2011).

Over the last 15 years, considerable progress has been made in the field of plant metabolic engineering for effective production of oilseeds rich in n-3 LC-PUFA (Ruiz-Lopez et al., 2015). This research and development has been encouraged by the relatively low production cost of transgenic

oilseeds as well as by the potential capability to adequately scale up for large volume applications (Petrie and Singh, 2011). The recent development of a DHA-containing oil extracted from *Camelina sativa* containing fish oil-like DHA levels of 12%, EPA levels of 3% and ALA levels of 29% presents a future alternative to meet the demand for n-3 LC-PUFA from aquaculture (Petrie et al., 2012, Petrie et al., 2014). This particular profile suggests promise as a substitute for fish oil because it theoretically supports the idea of enhancing efficiency of tissue deposition of n-3 LC-PUFA by sparing their deposition and promoting of ALA bioconversion. Novel DHA-containing oils extracted from transgenic plants are presently under development (Kitessa et al., 2014, Petrie et al., 2014), are only produced in limited quantities, and at the time of this study were unavailable in sufficient amount to perform a feeding trial. Thus, two oils were blended, tuna oil as a source of DHA (27% of total fatty acids), and flaxseed oil as a source of ALA (56% of total fatty acids), to obtain an oil blend that attempted to mimic the fatty acid profile of a novel *Camelina*-DHA oil. We tested the hypothesis that such a blended oil profile would result in fillet contents of n-3 LC-PUFA higher than those from current alternative oil-based diets and improve the efficiency of tissue deposition of n-3 LC-PUFA as compared to a fish oil-based diet. While EPA-containing oil extracted from transgenic *Camelina* has been produced (Ruiz-Lopez et al., 2014) and tested in Atlantic salmon (Betancor et al., 2015b) in the United Kingdom, the present study is the first attempt to perform a preliminary validation of an oil with high DHA and ALA profile in salmon, thereby aiming to assist in the further development and use in aquafeeds of novel DHA-containing oils with high ALA content.

A more precise control of feed composition requires both a broader screening and a more sensitive approach to understanding dietary induced physiological changes in fish. This can be achieved by being able to interpret changes in the liver proteome, as previously reported in response to other dietary modifications (Rodrigues et al., 2012, Ghisaura et al., 2014). The liver is a crucial site for fatty acid metabolism and body lipid homeostasis in Atlantic salmon and possesses other regulatory functions related to protein and carbohydrate metabolism, immunity or xenobiotic metabolism (Monroig et al., 2010, Betancor et al., 2015b). With regard to the specific use of sustainable ingredients in salmon aquafeeds, the liver proteome of Atlantic salmon has been studied in response to protein substitution (Sissener et al., 2010), but the relationship between the liver proteome and oil manipulation has not been investigated.

The objective of the present study was to test the effectiveness of an oil blend with high DHA and ALA content, as found in oil from transgenic *Camelina* seeds (Petrie et al., 2014), as a substitute for dietary fish oil in Atlantic salmon. Comparisons were made to a fish oil-based diet and a commercial-like oil blend containing fish oil and poultry oil as is currently used in aquafeeds for Atlantic salmon farmed in Tasmania (Australia). Our findings will primarily contribute towards further development and use of DHA-containing oil from transgenic plants as a sustainable solution for improving the nutritional value of salmon fillet. Secondly, knowledge of the liver proteome response to dietary oil manipulation is likely to provide useful insights into the regulatory mechanisms governing

lipid metabolism and other metabolism-relevant mechanisms that directly affect phenotypic traits in Atlantic salmon.

## 4.3. MATERIALS and METHODS

### 4.3.1. Experimental feeds

Three isonitrogenous and isolipidic experimental feeds were formulated to contain 240 g $kg^{-1}$ of lipid and 490 g $kg^{-1}$ of protein, varying only the lipid source: A 100% fish oil feed (FO), a blend of 20% fish oil and 80% poultry oil (FOPO) regarded as representative of current feeding practices in Tasmania, and a blend of 60% tuna oil and 40% flaxseed oil formulated to mimic the fatty profile of DHA-containing oil from *Camelina* (TOFX) (Table 4.1). Each feed included fish meal and yttrium oxide as a digestibility marker. Feeds were manufactured into 3 mm diameter pellets using a California Pellet Mill (CL-2, San Francisco, CA, USA), dried and stored at - 20 °C until use.

### 4.3.2. Feeding trial and sampling

The trial was conducted at the Institute for Marine and Antarctic Studies, University of Tasmania (Taroona, Tasmania, Australia)**.** All procedures implemented during this experiment were approved by the University of Tasmania Animal Ethics Committee (Investigation A0014093). Atlantic salmon smolts were sourced from a commercial hatchery (Petuna, Tasmania, Australia), randomly allocated in 12 × 500 L seawater tanks at an initial stocking density of 26 fish $tank^{-1}$ and acclimated for 28 days. Tanks were arranged in two independent six-tank partial recirculation systems, each equipped with a heat-exchanger, protein skimmer, drum filter, UV filter and biological filter. System seawater was continually supplied and progressively replaced twice daily, with tanks supplied at a rate of 8.5 L $min^{-1}$. Tanks were maintained at 15 °C water temperature, and at 12 h light:12 h dark photoperiod. Water quality parameters (pH, DO, nitrate and nitrite) were recorded daily and maintained within limits for Atlantic salmon (Wedemeyer, 1996). During acclimation, fish were fed a commercial feed (520 g $kg^{-1}$ of crude protein, 210 g $kg^{-1}$ of fat, and 21.9 MJ $kg^{-1}$ of GE; Skretting, Tasmania, Australia).

At the start of the trial, fish were anaesthetized (Aqui-S® 50 mg $L^{-1}$) (Javahery et al., 2012) and wet weight and fork length measured. A total of 24 fish, representative of the initial population, were euthanized (Aqui-S® 500 mg $L^{-1}$). Twelve whole carcasses were stored at -20 °C for initial chemical and fatty acid composition analyses. Dissected liver and dorsal muscle from the other twelve fish were frozen in liquid nitrogen and stored at -80 °C for initial proximate and fatty acid composition analyses. Four replicate tanks, two per recirculation system, were randomly assigned to each experimental diet or treatment. Feeds were provided at 1% fish body weight in two daily rations (0900 and 1700 h), and increased uniformly by 0.1% fish body weight every week. Uneaten feed was collected twice daily 10 min after feeding to accurately determine feed intake per tank. At 21-day intervals, all fish were anaesthetized and bulk weighed to monitor growth. After 89 days of growth, eight fish $tank^{-1}$ were

euthanized. Four whole carcasses were stored at -20 °C for final chemical and fatty acid composition analysis. Dissected dorsal muscle and liver from the other four fish were subsampled, frozen in liquid nitrogen, and stored at -80 °C for chemical and fatty acid composition and proteomic analyses. At the end of the trial (day 102), four fish per tank were anaesthetized and stripped for collection of faeces (Percival et al., 2001) and further proximate and fatty acid composition analyses. Fish were not fed for 24 h prior to being anaesthetized or euthanized.

### 4.3.3. Chemical composition

Tissues and whole carcasses sampled from the initial population were pooled, whereas tissues, whole carcasses and faeces from the final population were pooled on a per tank basis. Feeds, whole carcasses, tissues and faeces were freeze-dried to constant weight and milled to a fine powder. Dry matter was obtained by drying at 135°C for 2 h and ash content after incineration at 600°C for 2 h (AOAC, 1995). Crude protein was calculated after determination of total nitrogen by Kjeldahl analysis (Kjeltec$^{TM}$ 8100, Foss, Denmark), based on N x 6.25 (AOAC, 1995). Total lipid was obtained following overnight extraction using a modified Bligh and Dyer protocol (Alhazzaa et al., 2011), involving a single phase extraction using dichloromethane/methanol/water (1:2:0.8, v/v/v) followed by phase separation to yield a total lipid extract. Gross energy was measured by bomb calorimeter (6725 Semimicro, Parr, IL, USA). Analyses were performed in quadruplicate for experimental diets and initial sampling and in duplicate for final sampling. All analyses were corrected for dry matter.

### 4.3.4. Fatty acid analysis

An aliquot of the total lipid extract was trans-methylated in methanol/dichloromethane/hydrochloric acid (10:1:1, v/v/v) at 80 °C for 2 h. After addition of mQ water (1 mL), the mixture was extracted with hexane/dichloromethane (4:1, v/v) three times to obtain fatty acid methyl esters (FAME). FAME were made up to a known volume with internal injection standard (19:0 FAME, Nu-Chek Prep, Inc., MN, USA) and analysed by a 7890B gas chromatograph (Agilent Technologies, California, USA) equipped with a Supelco Equity$^{TM}$-1 fused silica capillary column (15 m × 0.1 mm i.d., 0.1 µm film thickness), flame ionization detector, split/splitless injector, and a 7683B auto sampler (Agilent Technologies, CA, USA). Helium was used as the carrier gas and samples were injected in splitless mode at an oven temperature of 120 °C. After injection, oven temperature was increased to 270 °C at 10 °C min$^{-1}$ and to a final temperature of 300 °C at 5 °C min$^{-1}$. Peaks were quantified with ChemStation software (Agilent Technologies, CA, USA) and initially identified using retention times from authentic and laboratory standards. Gas chromatography results are normally subject to an error of up to ±5 % of peak area. Absolute and relative values for each detected fatty acid were calculated from the areas of chromatogram peaks.

GC-mass spectrometric (GC-MS) analyses were performed on a Finnigan Trace GC-MS ultra Quadrupole GC-MS (ThermoQuest Trace DSQ, Thermo Electron Corporation, TX, USA). Data was

processed with ThermoQuest Xcalibur software (Thermo Electron Corporation, TX, USA). The GC fitted with an on-column injector and a capillary HP-5 Ultra column (50 m x 0.32 mm i.d., 0.17 μm film thickness, Agilent technologies, USA) of similar polarity to that described above. Individual components were identified using mass spectral data and by comparing retention time and MS data with those obtained for authentic and laboratory standards. A full procedural blank analysis was performed concurrent to the sample batch.

#### 4.3.5. Digestibility

Apparent digestibility (AD) was determined by assessing yttrium concentrations in experimental diets and faeces. Yttrium was analysed using inductively coupled plasma mass spectrometry (ELEMENT 2, Thermo Fisher Scientific Inc., MA, USA) following digestion with nitric acid and hydrogen peroxide as previously described (Ward et al., 2005). Apparent digestibility coefficients were calculated as (%) = 100 - (100 × ($Y_{diet}$ / $Y_{faeces}$) × ($X_{faeces}$ / $X_{diet}$)), where Y is the percentage of yttrium oxide and X is the percentage of a particular nutrient (Maynard, 1969).

#### 4.3.6. Fatty acid mass balance (FAMB)

A whole-body FAMB method was performed on the n-3 biosynthetic pathway to estimate the metabolic fate of ALA as previously described (Turchini and Francis, 2009).

##### 4.3.6.1. Liver preparation for proteomic analysis

##### 4.3.6.2. Protein extraction

Liver tissues from two fish (~ 50 mg each) from each tank (8 treatment$^{-1}$) were individually homogenized in Eppendorf tubes containing lysis buffer (7M urea, 2M thiourea, 50 mM pH 8 Tris) and protease inhibitor cocktail (Roche) using Tissue-Tearor homogenator (Biospec Products, OK, USA). Each extraction was performed for 18-24 h at 4 °C with overnight rotation. After removal of insoluble material by centrifugation, an aliquot was precipitated with 100% ethanol (9:1, v/v) overnight. Protein pellets were washed twice in 70 % ethanol and re-suspended in lysis buffer. Protein concentrations were estimated with Bradford Protein Assay (Bio-Rad) using plate reader (Synergy TMHT, BioTek, QL, Australia). Sampling pooling was used to reduce the effect of inter-individual variability relative to the biochemical differences between fish groups exposed to different farming conditions (Melis et al., 2014). Liver protein extracts were pooled by tank (n = 4) and the volumes adjusted with lysis buffer to achieve a concentration of 1 μg μL$^{-1}$ for each sample pool.

##### 4.3.6.3. Nano-liquid chromatography and tandem mass spectrometry (LTQ-Orbitrap XL)

Protein samples were trypsin-digested using standard procedures (Wilson et al., 2016) and analyzed by nanoLC-MS/MS using an LTQ-Orbitrap XL and Ultimate 3000 RSLCnano HPLC system (ThermoFisher Scientific, MA, USA). Tryptic peptides (~1 μg) were loaded onto a 20 mm x 75 μm

PepMap 100 trapping column (3 μm $C_{18}$) at 5 μl/min, using 98% water, 2% acetonitrile and 0.05% TFA. Peptides were separated at 0.3 μl/min on a 250 mm x 75 μm PepMap 100 RSLC column (2 μm $C_{18}$) held at 40ºC, using a stepped gradient from 97% mobile phase A (0.1% formic acid in water) to 50% mobile phase B (0.08% formic acid in 80% acetonitrile and 20 % water) comprising 3-10% B over 10 min, 10-40% B over 120 min, 40-50% B over 10 min, holding at 95% B for 10 min then re-equilibration in 3% B for 15 min. The LTQ-Orbitrap XL was controlled using Xcalibur 2.1 software in data-dependent mode and MS/MS spectra were acquired as described (Wilson et al., 2016).

#### 4.3.6.4. Database searching and criteria for protein identification

RAW files from the LTQ-Orbitrap were imported into MaxQuant software version 1.5.1.2 for peptide matching to MS/MS spectra and label-free protein quantification on the basis of median peptide intensity (LFQ) values (Cox et al., 2014). MS/MS spectra were searched against the Salmonidae database (http://uniprot.org/taxonomy/8030; 17,795 entries entries) using the Andromeda search engine. Default settings for protein identification were used, including a maximum of two missed cleavages, mass error tolerances of 20 ppm then 4.5 ppm for initial and main peptide searches, respectively, 0.5 Da tolerance for fragment ions, variable oxidation of methionine and fixed carbamidomethylation of cysteine. The false discovery rates (FDR) for peptide-spectrum matches and protein identification were both set to 0.01. MaxQuant output files of the complete peptide and protein-level mass spectrometry are provided in Supplemental Table 4.1 and 4.2, respectively.

#### 4.3.6.5. Calculations and statistical analysis

Standard formulae were used to assess growth, feed efficiency and biometrical data. Specific growth rate was calculated as SGR (% $d^{-1}$) = 100 × (ln $W_f$ / ln $W_i$) / d, where $W_f$ and $W_i$ are the final and initial weights (g) and d the number of days of the experiment. Feed efficiency ratio (g $g^{-1}$) was determined as FER = $W_g$ / FI, where $W_g$ is weight gain (g) over the feeding trial and FI is the total feed intake (g). Fulton's condition factor was calculated as k = W / $FL^3$, where W is fish weight (g) and FL is fork length (cm). Hepato-, whole gut-, and pyloric caeca- somatic indices were determined as HSI, GSI, PCSI = (TW / W) × 100, where TW is tissue weight (g) and W is fish weight (g). The % difference in fatty acid concentrations between diet and tissues was calculated as [(dietary fatty acid – tissue fatty acid) / dietary fatty acid] × 100.

Statistical analyses of growth, digestibility and chemical composition were performed using SPSS v22.0 software (IBM Corp., NY, USA). Data interpretation was based on one-way analysis of variance (ANOVA) at a significance level of 0.05. Data were checked with Levene's test to ensure normality and homogeneity of variance. Where significant differences were detected by ANOVA, data was subjected to Tukey-Kramer HSD post-hoc test. Results were expressed as mean ± standard error

(SEM) (n = 4) and different superscript letters within a row were used to denote significant differences ($p < 0.05$) among treatments.

The effect of two separate recirculation systems as a random factor was explored in the form of a randomized block design, resulting in only a significant difference in terms of feed intake ($p_{system}$ = 0.042, $F_{system}$ = 5.809) and consequently on final weight ($p_{system}$ = 0.004, $F_{system}$ = 15.629), SGR ($p_{system}$ = 0.011, $F_{system}$ = 10.955) and FER ($p_{system}$ = 0.011, $F_{system}$ = 11.006). Noting there was a balanced design and each feed was fed to duplicate tanks in each system. Accordingly, system was computed as random factor within the ANOVA analysis for all variables under study and will not be discussed further.

For statistical analysis of LTQ-Orbitrap mass spectrometry, the "ProteinGroups" output file generated by MaxQuant analysis of liver extracts was analysed in R (R Core Team, 2015) using the *limma* package (Ritchie et al., 2015). Proteins identified on the basis of a single matching peptide were excluded and only proteins detected in at least three biological replicates in any one treatment group were considered. The effect of diet was investigated by fitting a linear model with log2 protein group intensity as the response and diet and system as explanatory variables. All contrasts between different pairs of diets were extracted from this model. Prior to model fitting, intensity values were normalized using cyclic loess normalization (Bolstad et al., 2003) and the method of empirical array quality weights (Ritchie et al., 2006) was used to calculate sample reproducibility and down-weight less reproducible samples. After initial model fitting, empirical Bayes (Smyth, 2004) was used to calculate moderated test statistics and Benjamini Hochberg correction was applied to adjust p-values for multiple testing. Missing values for all remaining proteins were excluded from the analysis with degrees of freedom adjusted accordingly. Proteins differentially abundant at an adjusted p-value < 0.1 were selected for functional characterization. The salmonidae genes were first mapped to human orthologues using PANTHER (Mi et al., 2013) and then submitted to STRING v10 (Szklarczyk et al., 2015) for network enrichment analysis.

## 4.4. RESULTS

### 4.4.1. Chemical composition of feeds

The three experimental feeds were isonitrogenous (490.8 ± 0.86 g $kg^{-1}$ DM), isolipidic (242.8 ± 2.89 g $kg^{-1}$ DM) and isoenergetic (24.7 ± 0.03 MJ $kg^{-1}$) (Table 4.1). The fish oil-based feed (FO) was 2.5 and 2.8-fold higher in DHA and EPA, respectively, than the commercial-like feed (FOPO), resulting in similar DHA:EPA ratios of approximately of 1 (Table 4.2). The TOFX feed was similar to FO in DHA, but 3.5-fold lower in EPA, resulting in a DHA:EPA ratio of 3.4. The concentration of n-3 LC-PUFA in the TOFX feed was 1.6-fold lower than in the FO feed and 1.7-fold greater than in the FOPO feed. The TOFX feed was highest in ALA, with a concentration 11.4 and 18.7-fold greater than that of the FO and FOPO feeds, respectively. In both FO and TOFX feeds, polyunsaturated fatty acids (PUFA)

was the dominant fatty acid group and palmitic acid (16:0, PA) was the dominant fatty acid; in FOPO feed, monounsaturated fatty acids (MUFA) was the dominant fatty acid group with oleic acid (18:1n-9, OA) as the dominant fatty acid.

**Table 4.1. Ingredient and proximate composition of experimental feeds**

| | FO | FOPO | TOFX |
|---|---|---|---|
| *Ingredient composition (g kg$^{-1}$)* | | | |
| Fish meal[1] | 350 | 350 | 350 |
| Casein[2] | 146 | 146 | 146 |
| Wheat gluten[1] | 50 | 50 | 50 |
| Soy protein concentrate[1] | 116 | 116 | 116 |
| Fish oil[3] | 190 | 38 | - |
| Poultry oi[1] | - | 152 | - |
| Tuna oil[4] | - | - | 114 |
| Flaxseed oil[5] | - | - | 76 |
| Starch[6] | 74 | 74 | 74 |
| Vitamin mineral mix[7] | 3 | 3 | 3 |
| CMC[8] | 10 | 10 | 10 |
| Dicalcium Phosphate[9] | 6 | 6 | 6 |
| Stay-C[7] | 1.5 | 1.5 | 1.5 |
| Choline chloride[8] | 1 | 1 | 1 |
| Alpha-celullose[8] | 7.5 | 7.5 | 7.5 |
| Yttrium oxide[8] | 1 | 1 | 1 |
| Sipernat[10] | 40 | 40 | 40 |
| DL-Methionine[11] | 4 | 4 | 4 |
| | | | |
| *Proximate composition (g kg$^{-1}$ DM)* | | | |
| Dry matter (g kg$^{-1}$) | 906.5 | 897.2 | 902.0 |
| Crude protein | 489.7 | 492.5 | 490.2 |
| Total lipid | 237.9 | 247.9 | 242.5 |
| Ash | 111.2 | 111.0 | 109.0 |
| Gross energy (MJ kg$^{-1}$) | 24.7 | 24.7 | 24.6 |

FO, oil content of feed is 100% fish oil; FOCF, oil content of feed is 20% fish oil and 80% chicken fat; TOFX, oil content of feed is 60% tuna oil and 40% flaxseed oil
[1]Skretting Australia, Cambridge, TAS, Australia; [2]MP Biomedicals Australasia Pty., Seven Hills, NSW, Australia; [3]Chilean anchovy oil, Skretting Australia, Cambridge, TAS, Australia; [4]Clover Corporation/NuMega Lipids, Melbourne, VIC, Australia; [5]Kayban, Melbourne, VIC, Australia; [6]Starch Australasia, Lane Cove, NSW, Australia; [7]DSM, Wagga, NSW, Australia; [8]Sigma-Aldrich, Castle Hill, NSW, Australia; [9]ACE Chemical Company, Adelaide, SA, Australia; [10]Degussa, Frankfurt, Germany;[11]BEC Feed Solutions, Goodna, QLD, Australia.

**Table 4.2. Total fatty acid content (mg $g^{-1}$ lipid) and fatty acid composition of oils and feeds (as % total fatty acids)**

| | Oils | | | | Feeds | | |
|---|---|---|---|---|---|---|---|
| | FO | PO | TO | FX | FO | FOPO | TOFX |
| *Total fatty acid content* | 867.0 | 838.4 | 777.4 | 975.4 | 852.5 | 934.7 | 865.6 |
| *Fatty acid composition* | | | | | | | |
| 14:0 | 7.5 | 1.4 | 3.0 | 0.0 | 6.6 | 2.8 | 2.1 |
| 16:0 | 17.2 | 21.3 | 20.7 | 5.1 | 17.5 | 20.1 | 15.3 |
| 17:0 | 0.5 | 0.2 | 1.3 | 0.1 | 0.5 | 0.3 | 0.8 |
| 18:0 | 3.4 | 5.9 | 5.9 | 3.7 | 3.6 | 5.2 | 5.1 |
| Other SFA[1] | 1.1 | 0.4 | 2.3 | 0.5 | 1.1 | 0.6 | 1.5 |
| Total SFA | 29.8 | 29.2 | 33.3 | 9.5 | 29.3 | 29.1 | 24.8 |
| 16:1n-7 | 9.4 | 6.1 | 4.6 | 0.1 | 8.9 | 6.7 | 3.3 |
| 18:1n-7 | 3.2 | 2.6 | 2.3 | 1.0 | 3.2 | 2.9 | 2.0 |
| 18:1n-9 (OA) | 8.4 | 41.4 | 12.6 | 20.2 | 8.8 | 31.0 | 15.4 |
| 20:1n-9 | 0.8 | 0.5 | 1.2 | 0.3 | 0.8 | 0.6 | 0.8 |
| 22:1n-11 | 0.4 | 0.0 | 0.3 | 0.0 | 0.4 | 0.2 | 0.2 |
| Other MUFA[2] | 1.4 | 0.8 | 1.5 | 0.1 | 1.5 | 1.1 | 1.1 |
| Total MUFA | 23.6 | 51.5 | 22.4 | 21.7 | 23.6 | 42.4 | 22.8 |
| 18:2n-6 (LA) | 1.4 | 11.6 | 1.2 | 11.7 | 2.7 | 9.5 | 7.5 |
| 18:3n-6 | 0.3 | 0.1 | 0.1 | 0.0 | 0.3 | 0.2 | 0.1 |
| 20:3n6 | 0.2 | 0.1 | 0.1 | 0.0 | 0.2 | 0.1 | 0.1 |
| 20:4n-6 (ARA) | 0.8 | 0.3 | 1.8 | 0.0 | 0.6 | 0.5 | 1.1 |
| Other n-6 PUFA[3] | 0.7 | 0.2 | 2.0 | 0.1 | 0.7 | 0.4 | 1.3 |
| Total n-6 PUFA | 3.2 | 12.3 | 5.3 | 11.7 | 4.4 | 10.7 | 10.0 |
| 18:3n-3 (ALA) | 0.9 | 1.9 | 0.5 | 56.4 | 1.1 | 1.7 | 18.8 |
| 18:4n-3 (SDA) | 3.2 | 0.3 | 0.6 | 0.0 | 2.9 | 1.0 | 0.6 |
| 20:4n-3 (ETA) | 0.8 | 0.1 | 0.4 | 0.0 | 0.8 | 0.3 | 0.3 |
| 20:5n-3 (EPA) | 16.0 | 1.5 | 5.3 | 0.1 | 15.6 | 5.5 | 4.4 |
| 22:5n-3 (DPA) | 1.8 | 0.2 | 1.2 | 0.0 | 1.9 | 0.8 | 0.9 |
| 22:6n-3 (DHA) | 13.5 | 1.3 | 27.0 | 0.0 | 13.5 | 5.4 | 14.7 |
| Total n-3 LC PUFA[4] | 32.1 | 3.2 | 33.9 | 0.1 | 31.7 | 11.9 | 20.2 |
| Other n-3 PUFA[5] | 0.7 | 0.1 | 0.3 | 0.0 | 0.7 | 0.2 | 0.2 |
| Total n-3 PUFA | 36.8 | 5.5 | 35.2 | 56.5 | 36.4 | 14.9 | 39.8 |
| Total PUFA | 40.1 | 17.8 | 40.6 | 68.2 | 40.8 | 25.6 | 49.8 |
| n-3:n-6[6] | 11.3 | 0.4 | 6.6 | 5.8 | 8.4 | 1.4 | 4.0 |
| DHA:EPA[7] | 0.8 | 0.9 | 5.1 | 0.1 | 0.9 | 1.0 | 3.4 |
| ALA:LA[8] | 0.6 | 0.2 | 0.4 | 5.8 | 0.4 | 0.2 | 2.5 |
| EPA:ARA[9] | 21.1 | 5.1 | 2.9 | - | 27.0 | 11.0 | 4.1 |

Oils: FO, fish oil; PO, poultry oil; TO, tuna oil; FX, flaxseed oil; Feeds: FO, oil content of feed is 100% fish oil; FOPO, oil content of feed is 20% fish oil and 80% poultry oil; TOFX, oil content of feed is 60% tuna oil and 40% flaxseed oil

Data expressed as mean of four replicates per oil and feed

[1]Includes 15:0, 20:0, 21:0, 22:0, 23:0 and 24:0

[2]Includes 16:1n-5, 16:1n-9, 18:1n-5, 20:1n-7, 22:1n-9 and 24:1n-9

[3]Includes 20:2n-6, 22:5n-6, and 22:4n-6

[4]Includes 20:4n-3, 20:5n-3, 22:5n-3 and 22:6n-3

[5]Includes 21:5n-3, 24:6n-3 and 24:5n3

[6]n-3:n-6 ratio

[7]DHA:EPA ratio

[8]ALA:LA ratio

[9]EPA:ARA ratio

### 4.4.2. Growth performance and biometry

Feed intake was similar across treatments and fish fed the three diets doubled their initial wet weight (Table 4.3). Growth and biometry were not different in FO and FOPO fish, but were negatively affected in TOFX fish. Final weight was significantly lower ($p_{diet}$ = 0.042, $F_{diet}$ = 4.829) in TOFX fish as compared to FO or FOPO fish. SGR ($p_{diet}$ = 0.025, $F_{diet}$ = 6.046) and FER ($p_{diet}$ = 0.009, $F_{diet}$ = 8.893) were significantly lower in TOFX fish. Fork length was not different across treatments, but k was significantly ($p_{diet}$ = 0.033, $F_{diet}$ = 5.420) lower for TOFX fish than for FO or FOPO fish. HSI was not different across treatments. There were significant differences in GSI ($p_{diet}$ = 0.016, $F_{diet}$ = 7.302) and PCSI ($p_{diet}$ = 0.046, $F_{diet}$ = 4.624), with these in TOFX fish not different to those of FOPO fish, but lower than those of FO fish.

**Table 4.3. Growth performance, feed utilisation and biometry of Atlantic salmon smolt fed FO, FOPO and TOFX feeds over a 89 day period**

| | FO | FOPO | TOFX |
|---|---|---|---|
| Initial weight (g fish$^{-1}$) | 104.7 ± 0.83 | 105.4 ± 1.72 | 107.0 ± 1.92 |
| Final weight (g fish$^{-1}$) | 222.8 ± 5.47 ab | 229.3 ± 6.00 a | 214.1 ± 5.80 b |
| Feed intake (g fish$^{-1}$) | 130.2 ± 3.22 | 137.3 ± 5.42 | 131.4 ± 2.55 |
| SGR[1] 89d (%) | 0.85 ± 0.02 a | 0.87 ± 0.04 a | 0.78 ± 0.03 b |
| FER[2] | 0.91 ± 0.02 a | 0.90 ± 0.03 a | 0.81 ± 0.03 b |
| k[3] | 1.1 ± 0.03 a | 1.1 ± 0.02 a | 1.0 ± 0.01 b |
| HSI[4] (%) | 1.4 ± 0.17 | 1.2 ± 0.06 | 1.3 ± 0.11 |
| GSI[5] (%) | 10.4 ± 0.54 a | 9.3 ± 0.21 ab | 8.5 ± 0.15 b |
| PCSI[6] (%) | 4.9 ± 0.25 a | 4.4 ± 0.16 ab | 4.1 ± 0.11 b |

FO, oil content of feed is 100% fish oil; FOPO, oil content of feed is 20% fish oil and 80% poultry oil; TOFX, oil content of feed is 60% tuna oil and 40% flaxseed oil

Data expressed as mean ± SEM (n=4). Different letters within a row denotes significant differences among diets as determined by Tukey-Kramer HSD (p<

[1]Specific growth rate

[2]Feed efficiency ratio

[3]Condition factor

[4]Hepato-somatic index

[5]Gut-somatic index

[6]Pyloric caeca-somatic index

### 4.4.3. Digestibility

Dietary oil source did not have a significant effect on apparent digestibility (AD) of dry matter, crude protein, total lipid or gross energy. Digestibility values were on average 69.4 ± 0.09 % for dry matter, 89.3 ± 0.32 % for crude protein, 94.0 ± 0.08 % for lipid, and 80.6 ± 0.93 % for gross energy.

AD for fatty acids was significantly affected by dietary oil source, however, differences were smaller than 3% for most individual and classes of fatty acids (Appendix Table 4.1). AD of DHA was lowest ($p_{diet}$ = 0.004, $F_{diet}$ = 9.993) in TOFX fish, whereas AD of EPA and other n-3 LC-PUFA were not different across treatments. AD of ALA was lowest ($p_{diet}$ = 0.000, $F_{diet}$ = 30.110) in TOFX fish, and the same pattern was also followed for total n-3 PUFA ($p_{diet}$ = 0.002, $F_{diet}$ = 12.780) and total PUFA

($p_{diet}$ = 0.035, $F_{diet}$ = 5.275). AD of total MUFA was higher ($p_{diet}$ = 0.042, $F_{diet}$ = 4.819) in TOFX fish than in FO fish, but not different from FOPO fish, whereas AD of total SFA was not different across treatments and lower than for total PUFA and total MUFA.

### 4.4.4. Chemical composition of tissues and whole carcasses

Diet did not have a significant effect on chemical composition of tissues and whole carcasses. Contents in white dorsal muscle were on average 256.9 ± 1.74 g $kg^{-1}$ for dry matter, 803.9 ± 7.24 g $kg^{-1}$ DM for crude protein, 98.5 ± 3.49 g $kg^{-1}$ DM for lipid, and 73.0 ± 4.21 g $kg^{-1}$ DM for ash. Contents in liver were on average 258.9 ± 1.17 g $kg^{-1}$ for dry matter, 600.9 ± 7.51 g $kg^{-1}$ DM for crude protein, 153.4 ± 7.10 g $kg^{-1}$ DM for lipid, and 55.6 ± 1.13 g $kg^{-1}$ DM for ash. Contents in whole carcasses were on average 343.1 ± 2.20 g $kg^{-1}$ for dry matter, 498.8.9 ± 7.77 g $kg^{-1}$ DM for crude protein, 414.3 ± 7.52 g $kg^{-1}$ DM for lipid, and 83.9 ± 1.74 g $kg^{-1}$ DM for ash.

### 4.4.5. Fatty acid composition of tissues

Fatty acid profiles in muscle tissue were significantly different among dietary treatments (Table 4.4). The concentration of DHA was not different between FO and TOFX fish, and was higher ($p_{diet}$ = 0.000, $F_{diet}$ = 15.242) than that of FOPO fish. EPA was the highest ($p_{diet}$ = 0.000, $F_{diet}$ = 383.045) in FO fish, and higher in FOPO fish than in TOFX fish. Total n-3 LC-PUFA differed significantly across treatments ($p_{diet}$ = 0.000, $F_{diet}$ = 40.425), with the concentration in TOFX fish being higher than in FOPO fish and lower than in FO fish. ALA was highest ($p_{diet}$ = 0.000, $F_{diet}$ = 2319.401) in TOFX fish and not significantly different between FO and FOPO fish. The concentration of total n-3 PUFA in TOFX fish was higher ($p_{diet}$ = 0.000, $F_{diet}$ = 76.281) than in FOPO fish and was not different from FO fish, and the same pattern was followed in terms of absolute contents ($p_{diet}$ = 0.001, $F_{diet}$ = 15.889) (Figure 4.1). Total n-6 PUFA did not differ between TOFX and FOPO fish and was higher ($p_{diet}$ = 0.000, $F_{diet}$ = 114.197) than in FO fish. The resulting omega-3:omega-6 (n-3:n-6) ratio was significantly different ($p_{diet}$ = 0.000, $F_{diet}$ = 77.650), being higher in TOFX fish than in FOPO fish and lower than in FO fish. In TOFX and FO fish, PUFA was the dominant fatty acid group with DHA as the dominant fatty acid; in FOPO fish, MUFA was the dominant fatty acid group with OA as the dominant fatty acid.

**Table 4.4. Total fatty acid content (mg $g^{-1}$ lipid) and fatty acid composition (% total fatty acid) of dorsal white muscle of Atlantic salmon smolt fed FO, FOCF and TOFX feeds over a 89 day period**

| | Initial | FO | FOPO | TOFX |
|---|---|---|---|---|
| *Total fatty acid content* | 871.0 ± 48.81 | 858.8 ± 31.49 | 895.8 ± 17.31 | 882.7 ± 23.68 |
| *Fatty acid composition* | | | | |
| 14:0 | 2.7 ± 0.07 | 4.0 ± 0.32 a | 2.0 ± 0.14 b | 1.5 ± 0.09 b |
| 16:0 | 15.8 ± 0.05 | 17.7 ± 0.28 a | 18.4 ± 0.30 a | 15.2 ± 0.18 b |
| 17:0 | 0.3 ± 0.00 | 0.4 ± 0.01 b | 0.3 ± 0.00 c | 0.6 ± 0.01 a |
| 18:0 | 5.0 ± 0.06 | 4.7 ± 0.09 b | 5.1 ± 0.05 a | 5.3 ± 0.05 a |
| Other SFA[1] | 0.6 ± 0.01 | 0.7 ± 0.07 b | 0.5 ± 0.04 b | 0.9 ± 0.03 a |
| Total SFA | 24.3 ± 0.08 | 27.5 ± 0.41 a | 26.2 ± 0.40 a | 23.4 ± 0.32 b |
| 16:1n-7 | 6.0 ± 0.05 | 6.5 ± 0.34 a | 5.3 ± 0.16 b | 2.9 ± 0.04 c |
| 18:1n-7 | 3.3 ± 0.02 | 3.5 ± 0.07 a | 3.3 ± 0.02 b | 2.3 ± 0.03 c |
| 18:1n-9 (OA) | 29.3 ± 0.38 | 13.0 ± 0.68 c | 28.7 ± 0.72 a | 16.9 ± 0.53 b |
| 20:1n-9 | 1.5 ± 0.02 | 0.8 ± 0.10 b | 1.3 ± 0.03 a | 1.0 ± 0.06 b |
| 22:1n-11 | 0.4 ± 0.01 | 0.3 ± 0.02 a | 0.1 ± 0.01 b | 0.2 ± 0.02 b |
| Other MUFA[2] | 1.3 ± 0.02 | 1.5 ± 0.02 a | 1.3 ± 0.01 b | 1.2 ± 0.01 c |
| Total MUFA | 42.0 ± 0.42 | 25.6 ± 0.85 b | 40.0 ± 0.90 a | 24.4 ± 0.63 b |
| 18:2n-6 (LA) | 8.1 ± 0.07 | 2.9 ± 0.15 c | 7.5 ± 0.22 a | 6.4 ± 0.06 b |
| 18:3n-6 | 0.2 ± 0.00 | 0.1 ± 0.01 b | 0.2 ± 0.01 c | 0.1 ± 0.01 a |
| 20:3n-6 | 0.4 ± 0.01 | 0.3 ± 0.02 b | 0.5 ± 0.01 a | 0.2 ± 0.01 c |
| 20:4n-6 (ARA) | 0.8 ± 0.03 | 1.0 ± 0.04 ab | 0.9 ± 0.07 b | 1.1 ± 0.02 a |
| Other n-6 PUFA[3] | 0.9 ± 0.01 | 0.8 ± 0.04 b | 0.9 ± 0.02 b | 1.8 ± 0.35 a |
| Total n-6 PUFA | 10.4 ± 0.04 | 5.0 ± 0.19 b | 10.0 ± 0.14 a | 9.6 ± 0.35 a |
| 18:3n-3 (ALA) | 1.1 ± 0.02 | 1.0 ± 0.05 b | 1.3 ± 0.03 b | 12.8 ± 0.23 a |
| 18:4n-3 (SDA) | 1.0 ± 0.02 | 1.9 ± 0.13 a | 0.9 ± 0.02 b | 1.0 ± 0.05 b |
| 20:4n-3 (ETA) | 0.5 ± 0.00 | 0.8 ± 0.04 c | 0.4 ± 0.01 a | 0.6 ± 0.03 b |
| 20:5n-3 (EPA) | 3.5 ± 0.08 | 9.0 ± 0.18 a | 3.8 ± 0.16 b | 3.2 ± 0.11 c |
| 22:5n-3 (DPA) | 1.5 ± 0.01 | 3.3 ± 0.17 a | 1.4 ± 0.02 b | 1.4 ± 0.02 b |
| 22:6n-3 (DHA) | 12.1 ± 0.35 | 20.8 ± 1.14 a | 13.0 ± 1.11 b | 19.9 ± 0.85 a |
| Total n-3 LC PUFA[4] | 17.6 ± 0.44 | 33.9 ± 1.25 a | 18.6 ± 1.26 c | 25.0 ± 0.90 b |
| Other n-3 PUFA[5] | 0.7 ± 0.01 | 1.0 ± 0.06 a | 0.6 ± 0.07 b | 0.6 ± 0.05 b |
| Total n-3 PUFA | 20.5 ± 0.46 | 37.9 ± 1.16 a | 21.3 ± 1.21 b | 39.5 ± 0.87 a |
| Total PUFA | 30.9 ± 0.41 | 43.0 ± 1.05 b | 31.3 ± 1.07 c | 49.1 ± 0.55 a |
| n-3:n-6[6] | 2.0 ± 0.05 | 7.6 ± 0.44 a | 2.1 ± 0.15 c | 4.1 ± 0.20 b |
| DHA:EPA[7] | 3.4 ± 0.02 | 2.3 ± 0.10 c | 3.4 ± 0.18 a | 6.2 ± 0.18 b |
| ALA:LA[8] | 0.1 ± 0.00 | 0.4 ± 0.03 b | 0.2 ± 0.01 c | 2.0 ± 0.05 a |
| EPA:ARA[9] | 4.4 ± 0.08 | 9.2 ± 0.52 a | 4.5 ± 0.22 b | 2.8 ± 0.04 c |

FO, oil content of feed is 100% fish oil; FOPO, oil content of feed is 20% fish oil and 80% poultry oil; TOFX, oil content of feed is 60% tuna oil and 40% flaxseed oil

Data expressed as mean ± SEM (n=4). Different letters within a row denotes significant differences among diets as determined by Tukey-Kramer HSD ($p<0.05$)

[1]Includes 15:0, 20:0, 21:0, 22:0, 23:0 and 24:0
[2]Includes 16:1n-5, 16:1n-9, 18:1n-5, 20:1n-7, 22:1n-9 and 24:1n-9
[3]Includes 20:2n-6, 22:5n-6, and 22:4n-6
[4]Includes 20:4n-3, 20:5n-3, 22:5n-3 and 22:6n-3
[5]Includes 21:5n-3, 24:6n-3 and 24:5n3
[6]n-3:n-6 ratio
[7]DHA:EPA ratio
[8]ALA:LA ratio
[9]EPA:ARA ratio

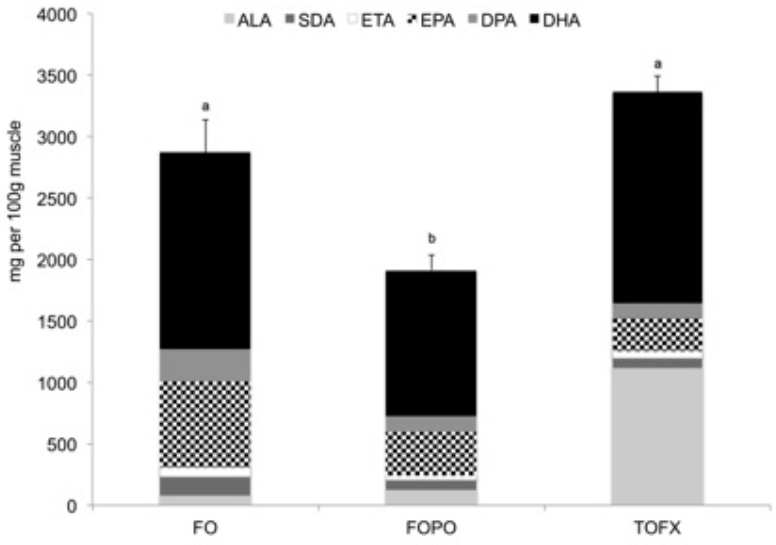


**Figure 4.1. Absolute content of total n-3 PUFA in white dorsal muscle of Atlantic salmon smolt fed FO, FOCF and TOFX feeds over a 89 day period.** Data expressed as mg per 100g (dry weight) of tissue. Values are means ± SEM (n = 4). Different letters denote significant differences ($p < 0.05$) among treatment means.

Fatty acid profiles in liver tissue were significantly different among dietary treatments (Table 4.5). DHA concentration in TOFX fish was not different from those of FO and FOPO fish, but was higher ($p_{diet} = 0.029$, $F_{diet} = 5.564$) in FO fish than in FOPO fish. EPA was the highest ($p_{diet} = 0.000$, $F_{diet} = 208.308$) in FO fish, and higher in FOPO fish than in TOFX fish. Total n-3 LC-PUFA in TOFX and FOPO fish was not different and was lower ($p_{diet} = 0.000$, $F_{diet} = 37.751$) than in FO fish. ALA was highest in TOFX fish and was not significantly different between FO or FOPO fish ($p_{diet} = 0.000$, $F_{diet} = 90.088$). Total n-3 PUFA in TOFX fish was higher ($p_{diet} = 0.000$, $F_{diet} = 44.773$) than in FOPO fish, but lower than in FO fish. Total n-6 PUFA in TOFX or FOPO fish was not different and lower ($p_{diet} = 0.000$, $F_{diet} = 408.187$) than in FO fish. The n-3:n-6 ratio differed significantly across treatments ($p_{diet} = 0.000$, $F_{diet} = 254.544$), with TOFX fish being higher than FOPO fish but lower than FO fish. PUFA was the dominant fatty acid group in all dietary treatments with DHA as the dominant fatty acid.

**Table 4.5. Total fatty acid content (mg $g^{-1}$ lipid) and fatty acid composition (% total fatty acid) of liver of Atlantic salmon smolt fed FO, FOCF and TOFX feeds over a 89 day period**

| | Initial | FO | FOPO | TOFX |
|---|---|---|---|---|
| *Total fatty acid content* | 698.9 ± 15.28 | 673.7 ± 35.11 | 676.0 ± 29.07 | 678.9 ± 8.44 |
| *Fatty acid composition* | | | | |
| 14:0 | 1.0 ± 0.12 | 1.9 ± 0.07 b | 1.0 ± 0.04 c | 0.9 ± 0.07 a |
| 16:0 | 18.1 ± 0.30 | 15.9 ± 0.22 | 14.7 ± 0.21 | 14.8 ± 0.85 |
| 17:0 | 0.3 ± 0.00 | 0.4 ± 0.02 b | 0.2 ± 0.01 b | 0.7 ± 0.07 a |
| 18:0 | 5.8 ± 0.06 | 6.8 ± 0.06 | 6.5 ± 0.18 | 7.3 ± 0.43 |
| Other SFA[1] | 0.4 ± 0.04 | 0.5 ± 0.02 | 0.8 ± 0.42 | 0.7 ± 0.03 |
| Total SFA | 25.6 ± 0.48 | 25.5 ± 0.19 | 23.3 ± 0.37 | 24.4 ± 1.31 |
| 16:1n-7 | 3.8 ± 0.07 | 2.9 ± 0.07 a | 2.6 ± 0.05 a | 1.7 ± 0.16 a |
| 18:1n-7 | 3.5 ± 0.05 | 3.5 ± 0.08 a | 3.2 ± 0.04 a | 2.4 ± 0.14 a |
| 18:1n-9 (OA) | 23.7 ± 0.23 | 12.1 ± 0.63 a | 22.4 ± 0.54 b | 15.2 ± 1.33 a |
| 20:1n-9 | 0.7 ± 0.03 | 1.4 ± 0.10 | 2.2 ± 0.18 | 1.3 ± 0.37 |
| 22:1n-11 | 0.2 ± 0.02 | 0.1 ± 0.01 | 0.0 ± 0.01 | 0.1 ± 0.03 |
| Other MUFA[2] | 2.1 ± 0.06 | 3.2 ± 0.09 b | 2.8 ± 0.05 a | 2.5 ± 0.09 a |
| Total MUFA | 33.9 ± 0.20 | 23.2 ± 0.79 b | 33.1 ± 0.60 a | 23.3 ± 1.79 b |
| 18:2n-6 (LA) | 6.5 ± 0.03 | 1.4 ± 0.03 b | 4.2 ± 0.16 a | 3.9 ± 0.31 a |
| 18:3n-6 | 0.3 ± 0.00 | 0.0 ± 0.00 b | 0.1 ± 0.02 a | 0.1 ± 0.02 ab |
| 20:3n-6 | 0.5 ± 0.00 | 0.4 ± 0.02 b | 1.2 ± 0.06 a | 0.4 ± 0.04 b |
| 20:4n-6 (ARA) | 2.1 ± 0.03 | 3.4 ± 0.09 ab | 3.4 ± 0.02 b | 4.0 ± 0.21 a |
| Other n-6 PUFA[3] | 0.8 ± 0.01 | 0.9 ± 0.06 b | 1.4 ± 0.07 ab | 2.0 ± 0.22 a |
| Total n-6 PUFA | 10.2 ± 0.04 | 6.1 ± 0.12 b | 10.3 ± 0.15 a | 10.3 ± 0.08 a |
| 18:3n-3 (ALA) | 0.9 ± 0.07 | 0.3 ± 0.02 b | 0.4 ± 0.03 b | 6.0 ± 0.56 a |
| 18:4n-3 (SDA) | 0.9 ± 0.02 | 0.5 ± 0.05 | 0.3 ± 0.03 | 0.5 ± 0.09 |
| 20:4n-3 (ETA) | 0.4 ± 0.00 | 0.6 ± 0.05 a | 0.3 ± 0.02 b | 0.7 ± 0.05 a |
| 20:5n-3 (EPA) | 4.7 ± 0.04 | 10.3 ± 0.20 a | 5.7 ± 0.28 b | 4.8 ± 0.17 c |
| 22:5n-3 (DPA) | 1.6 ± 0.02 | 3.2 ± 0.10 a | 1.5 ± 0.10 b | 1.4 ± 0.09 b |
| 22:6n-3 (DHA) | 19.6 ± 0.31 | 27.4 ± 0.57 a | 23.1 ± 0.59 b | 25.1 ± 1.23 ab |
| Total n-3 LC PUFA[4] | 26.2 ± 0.35 | 41.5 ± 0.68 a | 30.7 ± 0.85 b | 32.0 ± 1.22 b |
| Other n-3 PUFA[5] | 0.3 ± 0.04 | 0.4 ± 0.02 a | 0.3 ± 0.02 b | 0.3 ± 0.02 b |
| Total n-3 PUFA | 28.3 ± 0.36 | 42.7 ± 0.65 a | 31.6 ± 0.83 c | 38.8 ± 0.99 b |
| Total PUFA | 38.5 ± 0.32 | 48.9 ± 0.68 a | 41.9 ± 0.84 b | 49.1 ± 0.97 a |
| n-3:n-6[6] | 2.8 ± 0.05 | 7.0 ± 0.16 a | 3.1 ± 0.09 c | 3.7 ± 0.11 b |
| DHA:EPA[7] | 4.2 ± 0.04 | 2.7 ± 0.06 c | 4.0 ± 0.15 b | 5.3 ± 0.31 a |
| ALA:LA[8] | 0.1 ± 0.01 | 0.2 ± 0.01 b | 0.1 ± 0.00 c | 1.5 ± 0.05 a |
| EPA:ARA[9] | 2.2 ± 0.04 | 3.0 ± 0.03 a | 1.7 ± 0.08 b | 1.2 ± 0.08 c |

FO, oil content of feed is 100% fish oil; FOPO, oil content of feed is 20% fish oil and 80% poultry oil; TOFX, oil content of feed is 60% tuna oil and 40% flaxseed oil

Data expressed as mean ± SEM (n=4). Different letters within a row denotes significant differences among diets as determined by Tukey-Kramer HSD ($p<0.05$)

[1]Includes 15:0, 20:0, 21:0, 22:0, 23:0 and 24:0
[2]Includes 16:1n-5, 16:1n-9, 18:1n-5, 20:1n-7, 22:1n-9 and 24:1n-9
[3]Includes 20:2n-6, 22:5n-6, and 22:4n-6
[4]Includes 20:4n-3, 20:5n-3, 22:5n-3 and 22:6n-3
[5]Includes 21:5n-3, 24:6n-3 and 24:5n3
[6]n-3:n-6 ratio
[7]DHA:EPA ratio
[8]ALA:LA ratio
[9]EPA:ARA ratio

The difference between dietary and tissue concentrations of DHA, EPA, total n-3 LC-PUFA and ALA (in relation to dietary concentration) was significantly different among dietary treatments (Figure 4.2). The difference between diet and muscle DHA in the TOFX was not different from the FO treatment and was higher ($p_{diet}$ = 0.020, $F_{diet}$ = 16.067) than in the FOPO treatment. The difference between diet and muscle EPA in the TOFX treatment was not different from the FOPO treatment and was lower ($p_{diet}$ = 0.005, $F_{diet}$ = 11.258) than in the FO treatment. For both DHA and EPA, the same pattern of difference observed in muscle was found in liver, with the magnitude of difference being generally larger in liver than in muscle. The difference between diet and muscle n-3 LC-PUFA in the TOFX treatment was higher ($p_{diet}$ = 0.005, $F_{diet}$ = 11.117) than in the FOPO treatment and, although not significant, was numerically lower than in the FO treatment. The difference between diet and liver n-3 LC-PUFA in the TOFX treatment was higher ($p_{diet}$ = 0.000, $F_{diet}$ = 156.978) than in the FOPO treatment and lower than in the FO treatment. The difference between diet and muscle ALA in the TOFX treatment was not different from the FOPO treatment and was higher ($p_{diet}$ = 0.000, $F_{diet}$ = 26.726) than in the FO treatment, whereas the difference between diet and liver ALA did not differ across treatments.

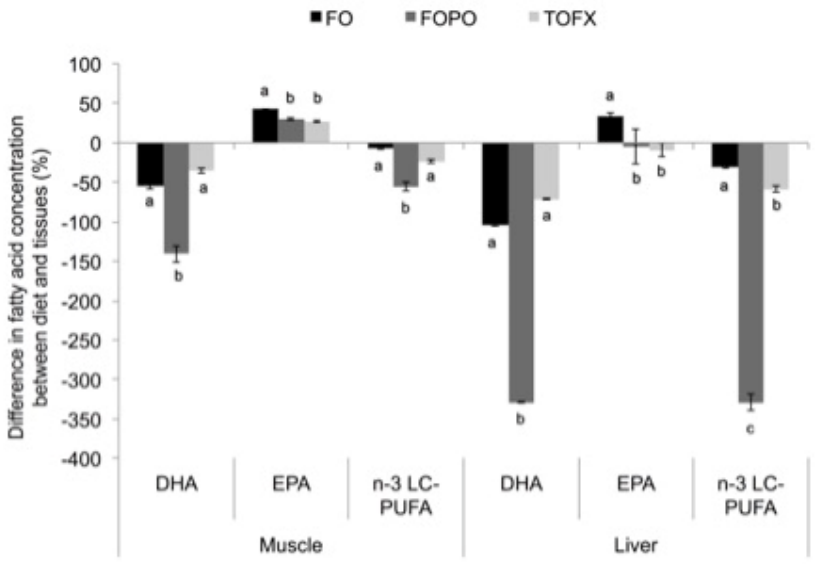


**Figure 4.2. Difference in dietary and tissue (white dorsal muscle and liver) concentrations of EPA, DHA, total n-3 LC-PUFA and ALA.** Data expressed as % difference in relation to dietary concentration. Values are means ± SEM (n = 4). Different letters denote significant differences ($p < 0.05$) among treatment means.

### 4.4.6. Fatty acid mass balance (FAMB)

FAMB suggested the metabolic fate of ALA was significantly affected by dietary oil source (Table 4.6). The net intake and absolute disappearance was highest ($p_{diet}$ = 0.000, $F_{diet}$ = 337.219) in TOFX fish. The retention efficiency in TOFX fish was not different from FOPO fish and was lower ($p_{diet}$ = 0.015, $F_{diet}$ = 6.000) than in FO fish. ALA bioconversion was not different across treatments.

**Table 4.6. Net intake (µmol fish$^{-1}$) and metabolic fate (µmol fish$^{-1}$ and % total net intake) of ALA in whole carcasses of Atlantic salmon smolt fed FO, FOPO and TOFX feeds over a 89 day period**

| | FO | FOPO | TOFX |
|---|---|---|---|
| Net intake[1] | 891.4 ± 20.95 c | 1677.0 ± 65.43 b | 15742.2 ± 363.78 a |
| Accumulated | 818.4 ± 52.28 b | 1271.0 ± 170.19 b | 9228.0 ± 695.77 a |
| % | 92.0 ± 5.96 a | 75.3 ± 8.09 ab | 58.4 ± 3.11 b |
| Disappeared | 73.1 ± 54.14 b | 406.0 ± 254.52 b | 6514.2 ± 356.80 a |
| % | 8.1 ± 6.00 b | 24.7 ± 16.17 ab | 41.6 ± 3.11 a |
| Oxidized | 0.0 ± 0.00 b | 401.5 ± 129.88 b | 6401.6 ± 378.75 a |
| % | 0.0 ± 0.00 b | 24.5 ± 8.23 a | 40.9 ± 3.23 a |
| Bioconverted | 73.1 ± 54.14 | 4.5 ± 4.46 | 112.6 ± 24.05 |
| % | 8.1 ± 6.00 | 0.2 ± 0.24 | 0.7 ± 0.14 |

FO, oil content of feed is 100% fish oil; FOPO, oil content of feed is 20% fish oil and 80% poultry oil; TOFX, oil content of feed is 60% tuna oil and 40% flaxseed oil

Data expressed as mean ± SEM (n=4). Different letters within row denotes significant differences among diets as determined by Tukey-Kramer HSD ($p<0.05$)

[1]Calculated as intake minus excretion

### 4.4.7. Liver proteomics

A total of 752 proteins were identified on the basis of two or more unique matching peptide sequences (Supplemental Table 4.3). Principal component analysis (PCA) showed maximum separation between TOFX and FO livers whereas FOPO livers showed considerable overlap with both FO and TOFX (Appendix Figure 4.1). Statistical comparison of intensity values identified significant differences between TOFX and FO livers, the results of which are shown on the volcano plot in Figure 4.3. On the basis of an adjusted $p < 0.1$, 11 proteins were differentially abundant, nine of which were up-regulated in TOFX livers and two in FO livers (Table 4.7). In addition, two proteins were detected only in TOFX livers and one was detected only in FO livers. Fold changes in differentially abundant proteins between FO and TOFX livers ranged from 4.3 to 1.3. Interaction network analysis of differentially abundant proteins revealed a significant enrichment for interacting proteins ($p = 0.027$) with three interactions (ALDH2-GSTT1, RAB1A-S61A1, S61A1-STT3A).

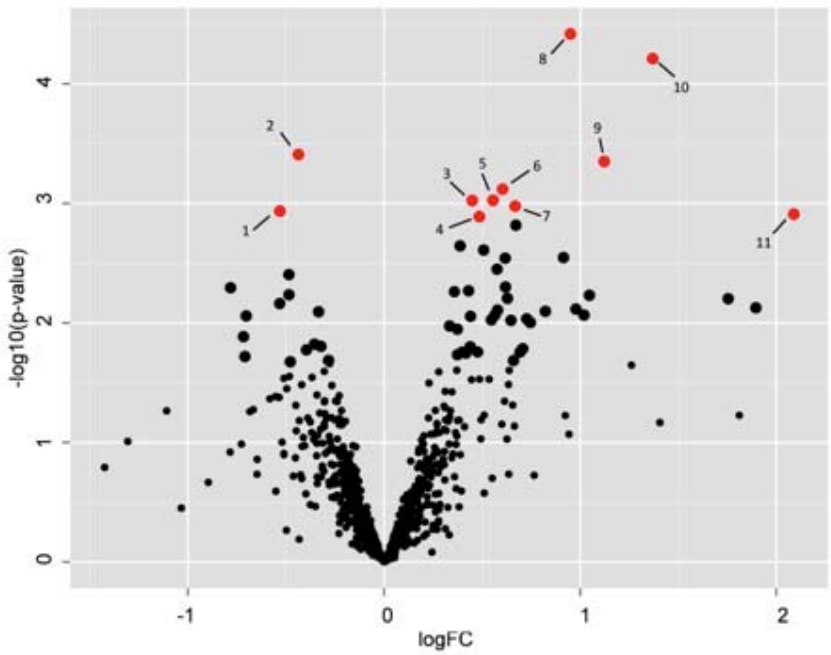


**Figure 4.3. Differences in protein abundance between FO and TOFX livers.** Volcano plot displaying differences of the pairwise comparison. Proteins found to be significantly (adjusted $p < 0.1$; $p < 0.05$) different between treatments are plotted in red and described in Table 7. Larger black circles represent those proteins significantly different at a lower stringent threshold (adjusted $p < 0.3$; $p < 0.05$).

**Table 4.7. Proteins significantly and differentially abundant in liver of Atlantic salmon smolt fed FO and TOFX feeds**

| # | Entry[1] | Gene name | Protein name | LFQ intensity[2] FO | LFQ intensity TOFX | Fold change[3] | No of unique peptides | p-value[4] | Adjusted p-value[5] |
|---|---|---|---|---|---|---|---|---|---|
| 1 | B5XCS5 | ATP5J | ATP synthase-coupling factor 6, mitochondrial | 1.21E+06 | 9.19E+05 | -1.3 | 2 | 0.001 | 0.094 |
| 2 | B5X2T3 | ALDH2 | Aldehyde dehydrogenase, mitochondrial | 7.53E+07 | 5.40E+07 | -1.4 | 25 | 0.000 | 0.089 |
| 3 | C0HBJ4 | RAB1A | Ras-related protein Rab-1A | 1.28E+06 | 1.80E+06 | 1.4 | 3 | 0.001 | 0.094 |
| 4 | B5X462 | GSTT1 | Glutathione S-transferase theta-1 | 4.55E+06 | 6.63E+06 | 1.5 | 9 | 0.001 | 0.094 |
| 5 | Q68S98 | | C type lectin receptor A | 7.19E+06 | 1.09E+07 | 1.5 | 2 | 0.001 | 0.094 |
| 6 | B5X313 | PAHX | Phytanoyl-CoA dioxygenase, peroxisomal | 5.37E+06 | 8.39E+06 | 1.6 | 11 | 0.001 | 0.094 |
| 7 | B5DG71 | H33 | Histone H3 | 4.69E+07 | 7.14E+07 | 1.5 | 2 | 0.001 | 0.094 |
| 8 | B5X180 | UD2A2 | UDP-glucuronosyltransferase 2A2 | 1.23E+06 | 2.43E+06 | 2 | 5 | 0.000 | 0.025 |
| 9 | B5X1I8 | STT3A | Dolichyl-diphosphooligosaccharide-protein glycosyltransferase subunit STT3A | 7.64E+05 | 1.76E+06 | 2.3 | 5 | 0.000 | 0.089 |
| 10 | B5X322 | S61A1 | Transport protein Sec61 subunit alpha isoform A | 1.01E+06 | 2.78E+06 | 2.8 | 4 | 0.000 | 0.025 |
| 11 | B5X2R4 | CP2M1 | Cytochrome P450 2M1 | 1.35E+06 | 5.59E+06 | 4.3 | 12 | 0.001 | 0.094 |
| 12 | B5X970 | PNPO | Pyridoxine-5-phosphate oxidase | 2.25E+06 | ND[6] | - | 4 | - | - |
| 12 | B5DGE7 | rtn4 | Reticulon | ND | 8.42E+05 | - | 3 | - | - |
| 14 | C0H9Z9 | F13A | Coagulation factor XIII A chain | ND | 1.15E+06 | - | 3 | - | - |

[1]UniProtKB from UniProt imported Salmo salar database
[2]Average LFQ intensity of non-normalised values (n = 4)
[3]Fold changes (TOFX vs. FO) in non-normalised LFQ intensities
[4]p value after pair-wise comparison
[5]False discovery rate adjustment using Benjamini-Hochberg correction. Reported proteins with values less than 0.1
[6]Not detected

## 4.5. DISCUSSION

### 4.5.1. Fatty acid profiles and nutritional implications

Novel oils extracted from transgenic oilseeds with fish oil-like proportions of DHA may provide the aquafeed industry with an option to enhance the nutritional quality of salmon flesh, as measured by the concentration of n-3 LC-PUFA, to the previous concentrations when marine oils were included in aquafeeds at higher amounts (Nichols et al., 2014). In the current study, a major comparison was between feeds formulated with fish oil (FO), as a historical standard, a current commercial-like oil blend (FOPO), and a blended oil reflecting the fatty acid profile of DHA-containing *Camelina* oil (TOFX) (Petrie et al., 2012, Petrie et al., 2014). The concentration of n-3 LC PUFA in white muscle was used as the most direct indication of how differences in dietary oil composition translated to differences in the product. This reached absolute contents of 2160 mg per 100 g (dry weight) in TOFX fish, and despite not being as high as in FO fish (2760 mg per 100 g), provided an extra 450 mg per 100 g muscle relative to the current industry FOPO diet.

Although the importance of ALA from a health perspective has been generally less reported relative to n-3 LC-PUFA, ALA is receiving interest as a nutraceutical supplement, based on reports of beneficial effects similar to those of n-3 LC-PUFA (Blondeau, 2016). ALA is a minor fatty acid in marine oils, however, it is highly and naturally present in *Camelina* and other transgenic oilseeds such as canola, reaching proportions of over 25% of the total fatty acids in oils extracted from transgenic seed (Petrie et al., 2014). Given the high relative level of ALA in the TOFX oil (22.8% total fatty acids), the muscle content of ALA was the highest in TOFX fish, with absolute levels of 1110 mg per 100 g of muscle in comparison with 86 and 123 mg per 100g of muscle in FO and FOPO fish, respectively. ALA is an essential fatty acid (EFA) for humans, and intake recommendations have been established at a minimum of 14 g weekly (Colquhoun et al., 2008).

Some evidence suggests that a balanced dietary n-3:n-6 ratio is positive in maintaining optimal human health (Simopoulos, 2008, Williams et al., 2011, Simopoulos, 2016). A ratio of 1:1-2 has been proposed as a target for adult human nutrition in relation to ratios of 1:10-20 in current western diets (Simopoulos, 2011). Consumption of fatty fish, and salmon in particular, is advised in western societies for the health benefits associated with high n-3 LC-PUFA content (NHMRC, 2006, EFSA, 2010). The intake of these, and n-3 in general rather than n-6, largely control the human dietary n-3:n-6 ratio (Deckelbaum, 2010). The n-3:n-6 ratio in TOFX fish (4.4), albeit not being as high as in FO fish (7.6), was markedly higher than in FOPO fish (2.2). This was directly attributed to the greater accumulation of both n-3 LC-PUFA and ALA, and implies that aquafeeds with high DHA and ALA content would enhance the nutritional quality of farmed salmon, and consequently the potential health benefits to the consumer relative to commercial blends containing a high proportion of poultry oil.

In aiming to enhance the current and future nutritional value of salmon, fish nutritionists should maximize deposition of n-3 LC-PUFA (Sanden et al., 2011). In the present study, the pattern of tissue

deposition in relation to diet was explored by the difference in the relative composition between diet and tissue as previously reported by Codabaccus et al. (2012) . In both muscle and liver, the difference in terms of DHA was similar between TOFX and FO treatments, whereas the difference in terms of EPA was lower in TOFX treatment than in FO treatment. This comparison indicates enhanced overall efficiency of tissue deposition of n-3 LC-PUFA in the TOFX treatment, as shown by the lower difference between dietary and tissue n-3 LC-PUFA in relation to FO; though the dissimilarity in this difference was only numerical in muscle, it was significant in liver These results were consistent with our hypothesis and with Codabaccus et al. (2012) , with the two studies collectively demonstrating that increasing the DHA:EPA ratio in relation to a fish-oil based diet promotes a tissue deposition "sparing effect" of n-3 LC-PUFA. This "sparing effect" is based on the mechanism of selective conservation of DHA; whereas n-3 LC-PUFA are normally oxidized for energy production if supplied in surplus, DHA is preferentially deposited as EPA is more extensively oxidized (Bransden et al., 2003, Stubhaug et al., 2007, Codabaccus et al., 2012). Accordingly, the DHA:EPA ratio of tissues increased in relation to diet, with this increase being less pronounced in TOFX fish than in FO fish due to the lower amount of EPA available for oxidation. Equally important, but unrelated to the DHA:EPA ratio, the sparing effect on n-3 LC-PUFA is also determined by absolute dietary concentration values as well as by the abundance of other SFA and MUFA that are more readily catabolized (Stubhaug et al., 2007, Budge et al., 2011, Codabaccus et al., 2012). This was clearly observed in FOPO treatment, which, despite a dietary DHA:EPA ratio similar to that of FO diet (~ 1), showed the lowest difference between dietary and tissue n-3 LC-PUFA concentration and consequently the highest efficiency in tissue deposition. Low n-3 LC-PUFA in FOPO diet promoted minimum EPA oxidation and maximum DHA deposition, with the more abundant oleic acid (18:1n-9, OA) and linoleic acid (18:2n-6, LA) in FOPO being used for energy production and thereby likely contributing to enhanced sparing and resultant tissue deposition of n-3 LC-PUFA.

Atlantic salmon hepatocytes possess the ability to desaturate and elongate $C_{18}$ fatty acids such as ALA to the long-chain and more unsaturated fatty acids (Tocher et al., 2003, Stubhaug et al., 2005, Zheng et al., 2005, Hixson et al., 2014a). Hence, we evaluated whether a high ALA concentration, as that occurring in the TOFX diet, might contribute to enhancing tissue deposition of n-3 LC-PUFA in relation to the FO diet by promoting desaturation and elongation. ALA in TOFX fish was largely accumulated and/or oxidized, and only minimally (< 1%) bioconverted, and thus no further ALA bioconversion was observed in relation to FO or FOPO fish. Limited or negligible bioconversion of ALA to n-3 LC-PUFA has been attributed to high availability of either substrate (ALA) or end-product (DHA). Whereas excessive $C_{18}$ fatty acids can affect elongation activities by limiting the ability of Δ6-desaturase to act on $C_{24}$ and consequently restraining DHA production (Senadheera et al., 2011, Thanuthong et al., 2011a), the high abundance of n-3 LC-PUFA, particularly DHA rather than EPA, can limit their possible biosynthesis due to an inhibitory effect on Δ6-desaturase and Elovl-2-like elongase enzymes (Zheng et al., 2004, Thomassen et al., 2012, Glencross et al., 2015). ALA

bioconversion has been reported to reach 25% of total net intake in Atlantic salmon fed a diet with full replacement of fish oil with *Camelina* oil (Hixson et al., 2014a, b); although ALA concentration was similar to that of TOFX diet (~ 20% total fatty acid), DHA was approximately 10-fold lower than in TOFX diet. Taken together, these findings suggest that DHA is likely more limiting than ALA itself in promoting ALA bioconversion in Atlantic salmon fed high DHA and ALA diets. The fairly high concentration of LA in the TOFX diet (7.5% total fatty acids) could also evoke competition between LA and ALA as substrates for the Δ6-desaturase enzyme, as proposed by composition-based studies in teleost (Vagner and Santigosa, 2011). However, this possibility is unlikely for salmonids, where more specific FAMB studies have shown that bioconversion of ALA is favored over LA due to the greater affinity of Δ6-desaturase for the n-3 pathway (Thanuthong et al., 2011b, Emery et al., 2013). In addition to bioconversion, an alternative pathway for ALA to improve tissue deposition of n-3 LC PUFA is through β–oxidation. ALA has a high rate of oxidation and, if present in large proportions, it is more readily utilized for energy production than other fatty acids (Bell et al., 2002, Stubhaug et al., 2007, Budge et al., 2011). For this hypothesis to be tested, a different approach using graded levels of ALA and constant levels of n-3 LC-PUFA and DHA:EPA ratio would be more suitable.

### 4.5.2. Growth and performance

Important commercial factors in the feeding of diets containing new alternative oils to Atlantic salmon also relate to the effects they have on growth performance. Several types of alternative oils have been investigated in salmon feeds, including vegetable (Bell et al., 2001, Bell et al., 2002, Bransden et al., 2003, Bell et al., 2004, Hixson et al., 2014a), animal fats (Emery et al., 2014, Emery et al., 2016) as well as new DHA and EPA containing oils (Miller et al., 2007b, Betancor et al., 2015b). These studies have collectively shown that growth is typically unaffected by dietary lipid source provided EFA requirements are met. All diets in the present study were formulated with non-defatted fishmeal that contained sufficient n-3 LC-PUFA to satisfy the EFA requirements of salmon (NRC, 2011). No difference in growth performance was found between FO fish and FOPO fish, as observed in previous studies with Atlantic salmon where fish oil was replaced at 50-75% with poultry oil (Codabaccus et al., 2012, Emery et al., 2014). However, TOFX diet had a small but negative effect on growth performance, feed efficiency, condition factor as well as in gut- and pyloric caeca- somatic indexes. Feed intake and digestibility of the main nutrients were not affected by dietary treatment, thus the observed detrimental effects were rather attributed to metabolic alterations resulting from impaired nutrient utilization and associated to the specific fatty acid composition of the TOFX diet. In this respect, n-3 LC PUFA increase β-oxidation in white fat preventing adipocyte hypertrophy and lipid accumulation (Parrish et al., 1990, Todorčević et al., 2008), and this effect is further supported by the adipogenic capacity of ALA (Oliva et al., 2013, Zhou et al., 2015). This offers a possible explanation for the observed lower gut somatic indices observed in TOFX fish and that could be the result of repressed visceral fat deposition.

### 4.5.3. Liver proteomics – potential oxidative stress responses

The liver proteome provides an informative biological matrix to study diet-induced changes and better understand the physiological basis of different phenotypic traits (Rodrigues et al., 2012, Ghisaura et al., 2014). Overall, our quantitative comparison among the liver proteomes found the most significantly different proteins between TOFX and FO diets, whereas comparisons between FOPO and FO, and between FOPO and TOFX, revealed no significant differences. This is consistent with the fact that TOFX and FO livers showed maximum separation by PCA analysis, whereas FOPO livers showed considerable overlap with both FO and TOFX.

Several proteins related to protein biogenesis in the ER and secretory protein trafficking were among the more highly up-regulated proteins in the TOFX livers, such as the alpha subunit of Sec6 (S61A1) and the glycosyltransferase subunit STT3A (STT3A). These two proteins were also increased in TOFX livers relative to FOPO livers, albeit at a less stringent cut-off (adjusted $p < 0.3$). However, the majority of protein alterations in the liver of TOFX fish were related to oxidative stress and detoxification pathways, such as changes in glutathione S-transferase theta-1 (GSTT1), cytochrome P450 2M1 (CP2M1) and mitochondrial aldehyde dehydrogenase (ALDH2). GSTT1 catalyzes the conjugation of the reduced form of glutathione to xenobiotic substrates and up-regulation has been associated to detoxification and increased protection against reactive molecules causing oxidative stress, such as hydrogen peroxides or aldehydes generated from lipid peroxidation (Pajaud et al., 2012, Singh et al., 2013). Glutathione transferases operate in parallel with aldehyde dehydrogenases and cytochrome P450 as hepato-protective mechanism against oxidative stress (Amunom et al., 2011), and thus alterations of these proteins have been observed in fish in response to temperature-induced (Ibarz et al., 2010) and xenobiotic-induced (Moore et al., 2003, Lee and Anderson, 2005) oxidative stress. ALDH2 was down-regulated in TOFX livers, following the trend previously reported in response to lipid peroxidation (Hjelle and Petersen, 1983, Wenzel et al., 2007) and/or to the presence of hydrogen peroxide (Towell and Wang, 1987, Oelze et al., 2011). In turn, expression of CP2M1 was increased, corroborating the role of cytochrome P450 as compensatory mechanism when the aldehyde dehydrogenase pathway is compromised (Amunom et al., 2011).

Lipid peroxidation-induced oxidative stress was also supported by changes in UD2A2 and pyridoxine-5-phosphate oxidase (PNPO). UD2A2 is the precursor of UDP-glucuronosyl transferase and up-regulation in TOFX fish may be related to the role of UDP-glucuronosyl transferase and glutathione transferases in preventing the propagation of lipid peroxidation (Olatunde Farombi, 2000, Rosland, 2014). PNPO catalyzes several reactions in the metabolism of vitamin B6, which has an antioxidant role by inhibiting lipid peroxidation or by serving as a coenzyme in the glutathione antioxidant defense system (Kannan and Jain, 2004). Increased oxidative stress has been reported in vitamin B6 deficient animals (Hsu et al., 2015), suggesting that down-regulation (to below detection limits) of PNPO in TOFX livers may have contributed to this response. Lipid peroxidation in TOFX livers was also evident

from changes in the abundance of several additional proteins (indicated by the data points labeled on Figure 4.3), albeit at a less stringent threshold for statistical significance (adjusted $p < 0.3$). Proteins that participate in the process of fatty acid oxidation (ECHA, NCPR, ECPH and PAHX) and in the subsequent antioxidant response (PRDX5, CATA and HBA), as well as oxidoreductases (CRYL1, AL3A2, G6PD) that catalyze these oxidative reactions, were mostly detected at elevated levels in the TOFX diet. Also related to fatty acid oxidation was the up-regulation of PAYH. This protein, along with AL3A2, catalyze the α-oxidation of phytanic acid, which is commonly found in high concentrations in tuna oil (van den Brink et al., 2004, Jansen and Wanders, 2006), thus reflecting the specific use of tuna oil in TOFX diet.

The alterations observed in the liver proteome of TOFX fish were indicative of oxidative stress related to peroxidative damage. This may be attributed to the fatty acid formulation of TOFX diet and related therefore to the impaired performance observed in fish. A possible cause was the higher total PUFA in TOFX diet, as evoked from the lower digestibility in relation to the other two diets, and from the lower tissue concentration in relation to feed. Increased susceptibility of fish to oxidative damage has been associated with excessive dietary PUFA; this relation is dependent on the efficacy of the antioxidant system (Kjær et al., 2008, ØStbye et al., 2011, Betancor et al., 2015a), and on the proportion that PUFA represents in the total dietary lipid (Olsen et al., 1999a, Glencross, 2009). In the present experiment, all feeds were considered on the safe side in terms of protection from peroxidation; feeds were evenly supplemented with antioxidant (vitamin C supplied by Stay-C, and vitamin E contained in the vitamin premix) in comparable amounts to those used in previous studies testing feeds with similar n-3 LC-PUFA content (Kjær et al., 2008, Betancor et al., 2015b), and stored at -20 °C. The PUFA concentration in TOFX diet were the highest across treatment and of 42 % of the total lipid, lying within the range (~ 37% - 50% total lipid) of previous data in salmonids that showed increased susceptibility to peroxidation through to growth reduction (Yu and Sinnhuber, 1979, Olsen and Henderson, 1997, Olsen et al., 1999a). In a more recent study with Atlantic salmon fed EPA-containing oil (ECO) extracted from transgenic *Camelina*, higher PUFA concentration (~55% of total lipid) did not affect growth (Betancor et al., 2015b), suggesting that fish vulnerability to oxidative damage is dependent on both the quality of the input oil(s), total PUFA and the balance among specific PUFA. While the formulation of TOFX and ECO diets were similar in terms of oil inclusion and nutrient composition, the most noticeable difference between the two were the high content of DHA and ALA (TOFX diet) versus a high content of EPA and LA (ECO diet). High dietary DHA amounts may induce oxidative stress and cause adverse effects on fish growth (Betancor et al., 2015a). The threshold from which such responses can be triggered in fish is broad (~ 10% - 36% total lipid) and correlated to the respective balance with the antioxidant supply (Stéphan et al., 1995, Kjær et al., 2008, ØStbye et al., 2011, Betancor et al., 2015a). DHA concentration in TOFX diet was high (12.7% total lipid), but close to those of the FO diet (11.5% total lipid), thus high DHA as unique cause of oxidative stress in TOFX fish was rather unlikely. In contrast, ALA concentration in TOFX diet was considerably high (~ 16%

of total lipid) in relation to the other diets and not far from dietary concentrations (~ 19% – 25% total lipid) that have previously resulted in growth detriment in Atlantic salmon (Ruyter et al., 2000, Hixson et al., 2014a). The negative effects of high dietary ALA have been associated with an increased susceptibility to lipid peroxidation and immunosuppression by a reduction in the antioxidant capacity provided by α-tocopherol (Wu et al., 1996, McGuire et al., 1997, Schimke et al., 1997). We therefore speculate that the antioxidant defense in TOFX fish could have been overwhelmed by the high dietary PUFA concentration, and in particular by the combination of high DHA and ALA. Despite the dietary inclusion of antioxidants, it is possible that the high susceptibility of DHA to oxidative damage was enhanced in our study by ALA-induced depression of the antioxidant system. The increased liver detoxification activities and small reduction in performance probably relates to these mechanisms. In this line, DHA is also more susceptible than EPA to oxidative breakdown (Song et al., 2000). The observed impaired performance in fish fed the TOFX diet in relation to those fed the EPA-containing oil diet, as in Betancor et al. (Betancor et al., 2015b), could be explained by the higher susceptibility of fish on the TOFX diet to oxidative damage and the need of extra antioxidant supplementation when fed oils rich in DHA and ALA. Future feeding trials should therefore consider the use of higher inclusion of antioxidants in feeds using such an oil blend, although considerably higher levels of antioxidants are present in *Camelina* and canola oils.

### 4.5.4. Conclusion

We used an oil blend with similar fatty acid composition to that of a DHA-containing oil extracted from transgenic *Camelina* (Petrie et al., 2014) and applied liver proteomics as an approach to gain insight into the future potential use of DHA-containing oils extracted from transgenic seeds, which are presently under development and limited in quantity. Such an oil blend (TOFX) clearly enhanced both the muscle content of n-3 LC-PUFA, DHA in particular, and the n-3:n-6 ratio as compared to a current commercial blend oil (FOPO) diet. From the human consumer perspective, DHA-containing oils extracted from transgenic terrestrial oil seeds are therefore potentially suitable as a means to improve the nutritional quality for consumers of salmon fillet. The particular fatty acid profile of the TOFX oil also promoted a more efficient deposition of n-3 LC-PUFA in muscle and liver in relation to a fish oil-based diet; this was attributed to less inefficient utilization, in particular as occurs via β-oxidation, of n-3 LC-PUFA due to the higher DHA:EPA ratio, rather than to bioconversion from the high**er** content of the ALA precursor. The TOFX oil triggered an oxidative stress response that was likely associated to impaired growth. This draws attention to the necessity of considering the balance between total and specific PUFA and antioxidant levels present in the diet and the possible need for supplementation in order to protect PUFA from oxidative damage in both the feed and in the fish**.** Notwithstanding, we also note that unlike the oil blend used in this study, the pending transgenic terrestrial oils do contain elevated levels of naturally occurring antioxidants including carotenes and

tocopherols (CSIRO, unpublished data). This aspect will need further consideration for the future inclusion of DHA-containing oils extracted from transgenic *Camelina* or canola in salmon aquafeeds.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD004114

# CHAPTER 5

# Liver proteome response of pre-harvest Atlantic salmon following exposure to elevated temperature

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## 5.1. ABSTRACT

Atlantic salmon production in Tasmania (Southern Australia) occurs near the upper limits of the species thermal tolerance. Summer water temperatures can average over 19°C over several weeks and have negative effects on performance and health. Liver tissue exerts important metabolic functions in thermal adaptation. With the aim of identifying mechanisms underlying liver plasticity in response to chronic elevated temperature in Atlantic salmon, label-free shotgun proteomics was used to explore quantitative protein changes after 43 days of exposure to elevated temperature. A total of 277 proteins were differentially (adjusted p-value <0.05) expressed between the control (15°C) and elevated (21°C) temperature treatments. As identified by Ingenuity Pathway Analysis (IPA), transcription and translation mechanisms, protein degradation via the proteasome, and cytoskeletal components were down-regulated at elevated temperature. In contrast, an up-regulated response was identified for NRF2-mediated oxidative stress, endoplasmic reticulum stress, and amino acid degradation. The proteome response was paralleled by reduced fish condition factor and hepato-somatic index at elevated temperature. The present study provides further evidence of the interplay among different cellular machineries in a scenario of heat-induced energy deficit and oxidative stress, and refines present understanding of how Atlantic salmon cope with chronic exposure to temperature near the upper limits of thermal tolerance.

## 5.2. INTRODUCTION

A major impact of climate change on fisheries and aquaculture is the increased seawater temperature (De Silva and Soto, 2009). Aquatic ectotherms species in the temperate areas are particularly vulnerable given the high warming rate of this area (Neuheimer et al., 2011). In Tasmania (Southern Australia), seawater Atlantic salmon is produced near the upper limits of thermal tolerance (Miller et al., 2006). Post-smolt Atlantic salmon are generally considered to have an optimum temperature range of 13-15°C for growth and an upper critical range of 22-33°C. (Jonsson and Jonsson, 2009). While, in Tasmania, the sea surface temperature averages over 19°C for several weeks during the summer period (IMOS-OceanCurrent, unpublished data) and further increases are projected (Oliver et al., 2014). Sea-caged Atlantic salmon cannot escape the increased surface water temperatures by vertical migration, thus risk long-term exposure to heat stress and physiological challenges that both impair production efficiency and raise welfare issues (Battaglene et al., 2008).

Heat stress co-occurs with hypoxia, and although oxygen limited thermal tolerance is of major importance in the fish physiological response (Portner and Knust, 2007), the temperature-dependent response occurs independently of water oxygen saturation (Hevroy et al., 2012, Kullgren et al., 2013). Any temperature rise increases the metabolic rate and consequently the maintenance of energy requirements, leading to a state of metabolic remodeling to compensate for increased energy demand (Jobling, 1994). This response is dependent on the species' thermal tolerance (eurythermals vs. stenothermals) and the exposure regime (acute vs. short-term vs. long-term) (Logan and Buckley, 2015). Individual tissues show highly divergent responses to thermal stress that are likely related to their physiological role in the body (Logan and Buckley, 2015). The liver tissue is the central hub for the regulation of nutrient metabolism and detoxification, and thus it constitutes an excellent target to characterize mechanisms of acclimation to chronic elevated temperature. To our knowledge, the liver molecular plasticity of Atlantic salmon to chronic elevated temperature is limited to the study of the liver transcriptome (Olsvik et al., 2013). The most notable findings of this study were reduced protein synthesis and increased xenobiotic metabolism in fish held at 19°C. Shotgun proteomics can provide an unprecedented view of the liver response of this species to chronic heat stress, and offers potential to unravel adaptive physiological mechanisms that can be only postulated by the transcriptome. In addition, and in relation to previous fish proteomics research, the liver response to thermal stress has been only examined using the gel-based approach (Ibarz et al., 2010, Mahanty et al., 2016). Shotgun proteomics can extend the range of quantifiable proteins and provide a more detailed characterization of the affected mechanisms that are common to different species and thermal stresses.

Here we explored the long-term exposure to elevated temperature on the molecular response in liver tissue of pre-harvest Tasmanian Atlantic salmon using label-free shotgun proteomics. We compared two water temperatures, 15°C and 21°C, under the same oxygen saturation levels and after 43 days of exposure. Quantitative changes in the protein expression pattern can contribute to the

mechanistic understanding of how Atlantic salmon specifically and fish generally cope with chronic elevated temperatures. This study expands fundamental and applied information about fish nutrition under limiting environmental conditions previously conducted in Tasmania (Katersky and Carter, 2005, Katersky and Carter, 2007b, a, Carter et al., 2008). These findings may be also of value for the aquaculture industry in the further development of dietary formulations for Atlantic salmon specific to the summer period.

## 5.3. MATERIALS and METHODS

### 5.3.1. Growth trial and sampling

The trial was conducted at the Experimental Aquaculture Facility (EAF) of the Institute for Marine and Antarctic Studies, University of Tasmania (Taroona, Tasmania, Australia) in accordance with University of Tasmania Animal Ethics (Investigation A0015208). Atlantic salmon post-smolt (average weight ± STD: 961 ± 172 g) were sourced from a commercial hatchery (Huon Aquaculture, Tasmania, Australia), randomly allocated amongst 6 × 2500 L circular tanks at an initial stocking density of 19 fish $tank^{-1}$ and acclimated for 38 days. Each tank had an independent recirculation system equipped with a heat-exchanger, protein skimmer, drum filter, UV filter and biological filter. Seawater was continually supplied and progressively replaced 1.5 times $hour^{-1}$ with 10% new water exchange $day^{-1}$. Water temperature was maintained at 15 °C during acclimation. Photoperiod was maintained at 12 h light:12 h dark. Water quality parameters (DO, pH, nitrate and nitrate) were recorded daily and maintained within limits for Atlantic salmon (Wedemeyer, 1996); dissolved oxygen (DO) was maintained at 101 ± 0.8% and pH at 7.8 ± 0.0. Salinity was 34 ppt throughout the experiment. Fish were fed a commercial diet (Optiline 8 mm, Skretting, Tasmania, Australia) in excess four times $day^{-1}$ with automatic feeders, and uneaten pellets were collected after termination of each meal to calculate daily feed intake. The feed contained 42% crude protein, 29% total lipid, and 21.4 MJ $kg^{-1}$ digestible energy.

In order to examine the response to elevated chronic temperature, fish were exposed to two temperatures, 15°C and 21°C. Temperature in triplicate tanks was steadily increased (0.5 °C $day^{-1}$) over 13 days until it reached 21°C, and then maintained for 43 days until the end of the experiment. At the beginning of the experiment, fish were anaesthetized (Aqui-S® 50 mg $L^{-1}$) (Javahery et al., 2012) and benchmarked (wet weight, fork length and skin/fin condition). At the end of the experiment fish were euthanized (Aqui-S® 500 mg $L^{-1}$) and re-measured for wet weight and fork length. Liver was dissected and weighed, immediately frozen in liquid nitrogen, and stored at -80 °C for proteomic analysis. Growth rate was correlated with condition factor (k) (C.G. Carter personal observation) and fish with $k < 1.2$ were not sampled, otherwise livers were randomly sampled (three $tank^{-1}$, nine $treatment^{-1}$. The effect of elevated temperature on growth biometry and liver protein expression was therefore assessed on an individual basis (n = 9).

### 5.3.2. Liver preparation for proteomic analysis

#### 5.3.2.1. Protein extraction

Liver tissues (n = 9; ~ 60 mg each;) were individually homogenized in Eppendorf tubes containing lysis buffer (7M urea, 2M thiourea, 50 mM pH 8 Tris) and protease inhibitor cocktail (Roche) using Tissue-Tearor homogenator (Biospec Products, OK, USA). Each extraction was performed for 18-24 h at 4 °C with overnight rotation. After removal of insoluble material by centrifugation, an aliquot was precipitated with 100% ethanol (9:1, v/v) overnight. Protein pellets were washed twice in 70 % ethanol and re-suspended in lysis buffer. Protein concentrations were estimated with Bradford Protein Assay (Bio-Rad) using plate reader (Synergy TMHT, BioTek, QL, Australia) and the volumes were adjusted with lysis buffer to achieve a concentration of 1 μg $\mu L^{-1}$ for each extract.

#### 5.3.2.2. Nano-liquid chromatography and tandem mass spectrometry (LTQ-Orbitrap XL)

Protein samples were trypsin-digested using standard procedures (Wilson et al., 2016) and analyzed by nanoLC-MS/MS using an LTQ-Orbitrap XL and Ultimate 3000 RSLCnano HPLC system (ThermoFisher Scientific, MA, USA). Tryptic peptides (~1 μg) were loaded onto a 20 mm x 75 μm PepMap 100 trapping column (3 μm $C_{18}$) at 5 μl/min, using 98% water, 2% acetonitrile and 0.05% TFA. Peptides were separated at 0.3 μl/min on a 250 mm x 75 μm PepMap 100 RSLC column (2□μm $C_{18}$) held at 40ºC, using a stepped gradient from 97% mobile phase A (0.1% formic acid in water) to 50% mobile phase B (0.08% formic acid in 80% acetonitrile and 20 % water) comprising 3-10% B over 10 min, 10-40% B over 180 min, 40-50% B over 10 min, holding at 95% B for 10 min then re-equilibration in 3% B for 15 min. The LTQ-Orbitrap XL was controlled using Xcalibur 2.1 software in data-dependent mode and MS/MS spectra were acquired as described (Wilson et al., 2016).

#### 5.3.2.3. Database searching and criteria for protein identification

RAW files from the LTQ-Orbitrap were imported into MaxQuant software version 1.5.1.2 for peptide matching to MS/MS spectra and label-free protein quantification on the basis of median peptide intensity (LFQ) values (Cox et al., 2014). MS/MS spectra were searched against the Salmonidae database (http://uniprot.org/taxonomy/8030; 17,795 entries entries) using the Andromeda search engine. Default settings for protein identification were used, including a maximum of two missed cleavages, mass error tolerances of 20 ppm then 4.5 ppm for initial and main peptide searches, respectively, 0.5 Da tolerance for fragment ions, variable oxidation of methionine and fixed carbamidomethylation of cysteine. The false discovery rates (FDR) for peptide-spectrum matches and protein identification were both set to 0.01. MaxQuant output files of the complete peptide and protein-level mass spectrometry are provided in Supplemental Tables 5.1 and 5.2, respectively.

### 5.3.3. Calculations and statistical analysis

Standard formulae were used to assess growth biometrical data. Fulton's condition factor was calculated as $k = W / FL^3$, where W is fish wet weight (g) and FL is fork length (cm). Hepato-somatic index was determined as $HSI = (LW / W) \times 100$, where LW is liver weight (g) and W is fish wet weight (g).

Statistical analyses of biometrical data was performed using R software (R Core Team, 2016). Individual fish data was analysed using the Generalized Estimating Equations (GEE) model to control the cluster (tank) correlation derived from the sampling of individuals from different tanks within each treatment (Højsgaard et al., 2005). Tank and fish nested within tank were considered random variables. The Wald test was used to detect significant differences ($p < 0.05$) between treatments and results were expressed as mean ± standard error (SEM) ($n = 9$).

For statistical analysis of LTQ-Orbitrap mass spectrometry, the "ProteinGroups" output file generated by MaxQuant analysis of liver extracts was analysed in R (R Core Team, 2016) using the *limma* package (Ritchie et al., 2015). Proteins identified on the basis of a single matching peptide were excluded and only proteins detected in at least six out of nine biological replicates in any one treatment group were considered. The effect of temperature was investigated by fitting a linear model with log2 protein group intensity as the response and including tank and fish nested to tank as explanatory random variables. Prior to model fitting, intensity values were normalized using cyclic loess normalization (Bolstad et al., 2003) and the method of empirical array quality weights (Ritchie et al., 2006) was used to calculate sample reproducibility and down-weight less reproducible samples. After initial model fitting, empirical Bayes (Smyth, 2004) was used to calculate moderated test statistics and Benjamini Hochberg correction was applied to adjust p-values for multiple testing. Missing values for all remaining proteins were excluded from the analysis with degrees of freedom adjusted accordingly. To gain further insight into the potential mechanisms of the effects of elevated temperature, differentially (adjusted p-value < 0.05) expressed proteins were selected for Ingenuity Pathway Analysis (IPA). The salmonidae genes were first mapped to human orthologues using PANTHER (Mi et al., 2013), and then gene symbols and the corresponding fold change in protein expression were submitted to IPA for identification of canonical and toxicity pathways and mapping of interaction networks.

## 5.4. RESULTS

### 5.4.1. Biometric indices and feed intake

Elevated temperature had a significant and negative effect on k and HSI, but this effect was reflected only numerically in final weight (Table 5.1). Feed intake (measured by tank) was not significantly affected by elevated temperature. The trend in biometric indices on selected individuals was similarly reflected in tank-based comparisons (not reported).

**Table 5.1. Biometric indices of pre-harvest Atlantic salmon (selected on the basis of k[1] > 1.2) held at 15°C and 21°C**

| | 15°C | 21°C | Test (p-value, *Wald*[2]) |
|---|---|---|---|
| Final wet weight (g) | 2367.1 ± 140.8 | 2064.9 ± 104.10 | 0.140, - |
| Final length (mm) | 523.9 ± 7.8 | 565.0 ± 13.85 | 0.756, - |
| k[1] | 1.6 ± 0.06 | 1.4 ± 0.07 | 0.028, 4.834 |
| HIS[3] | 1.2 ± 0.0 | 1.0 ± 0.07 | 0.000, 14.228 |

Data expressed as mean ± SEM (n=9). Each replicate represented by an individual fish
[1]Condition factor
[2]Wald estimator only reported when p-value <0.05
[3]Hepato-somatic index

### 5.4.2. Liver proteomics

A total of 842 proteins were identified on the basis of two or more unique matching peptide sequences and presence in at least six biological replicates in any of the treatment groups (Supplemental Table 5.3). Multidimensional scaling (MDS) showed maximum separation between temperature treatments (Figure 5.1A). On the basis of an adjusted p-value < 0.05, comparison of intensity values identified differences in 276 proteins, which are shown on the volcano plot in Figure 5.1B. Of these proteins, 89 and 187 proteins were up-regulated and down-regulated in 21 °C livers relative to 15 °C livers, respectively, with fold changes ranging from 1.2 to 5.4. Differentially abundant proteins showing fold changes > 2 are listed in Table 5.2. One protein, ferritin (FTL), showed a significant (adjusted p-value < 0.05) degree of correlation (Pearson correlation coefficient = 0.92) with the measured HSI (Figure 5.2).

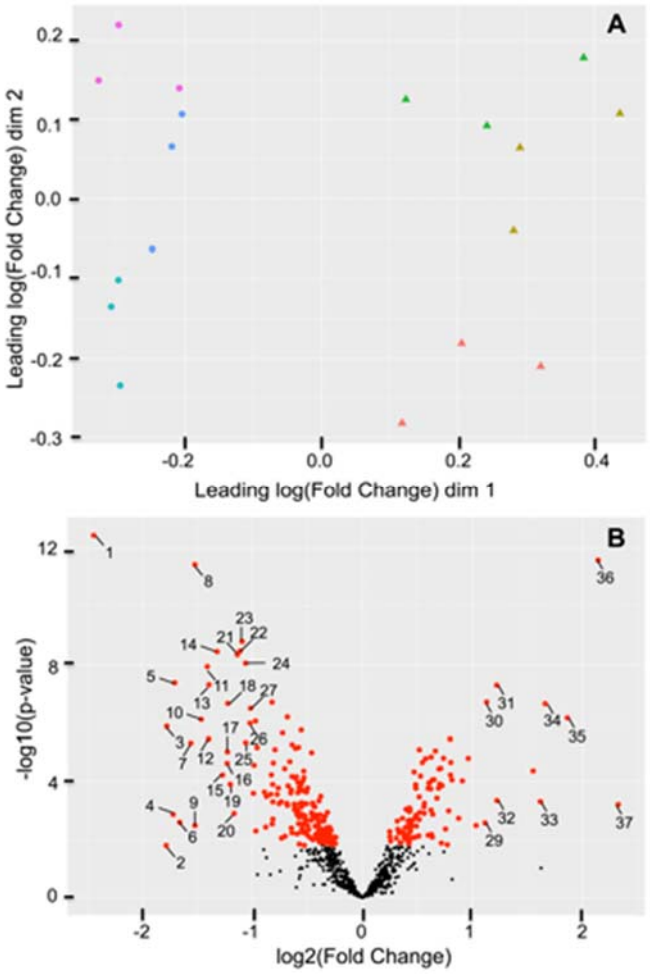


**Figure 5.1. Multidimensional scaling (MDS) (A) and volcano plot (B) of the liver proteome profile of Atlantic salmon held at 15°C and 21°C.** A) Data points in the MDS are plotted with symbols (•: 15°C; ▲: 21°C), representing temperature treatments, and colors, representing tank allocation. B) Red dots in the volcano plot represent proteins to be significantly different (adjusted p-value <0.05). Numbered dots refer to proteins displaying fold changes > 2 as described in Table 5.2.

**Table 5.1. List of differentially abundant proteins with fold changes > 2 in liver of pre-harvest Atlantic salmon held at 15°C and 21°C.**

| # | Protein name *(Entry name)* | Fold change 21°C vs. 15°C | Unique peptides | adj. p-value[1] | Gene name | Human orthologue |
|---|---|---|---|---|---|---|
| 1 | Acyl-coenzyme A oxidase *(C0H935)* | -5.43 | 14 | <0.001 | ACOX3 | ACOX3 |
| 2 | Leukocyte cell-derived chemotaxin 2 *(B5XCD7)* | -3.44 | 6 | 0.050 | LECT2 | LECT2 |
| 3 | Betaine-homocysteine methyltransferase *(B5DGE7)* | -3.43 | 13 | <0.001 | bhmt | BHMT |
| 4 | 5'-nucleotidase (EC 3.1.3.5) *(B5DGD0)* | -3.29 | 3 | 0.009 | 5NT3L | NT5C3A |
| 5 | Uricase (EC 1.7.3.3) (Fragment) *(Q3S563)* | -3.26 | 16 | <0.001 | | |
| 6 | Cytochrome P450 2M1 *(B5X2R4)* | -3.16 | 7 | 0.014 | CP2M1 | |
| 7 | Tubulin folding cofactor B *(B5X4J7)* | -2.95 | 9 | <0.001 | TBCB | TBCB |
| 8 | Elongation factor 2 *(C0H9N2)* | -2.87 | 9 | <0.001 | EF2 | EEF2 |
| 9 | Sulfotransferase (EC 2.8.2.-) *(B5X695)* | -2.87 | 10 | 0.016 | ST2S2 | ST2S2 |
| 10 | Pyruvate kinase (EC 2.7.1.40) *(C0HBL8)* | -2.76 | 28 | <0.001 | KPYK | KPYK |
| 11 | High mobility group protein B3 *(C0HBT7)* | -2.66 | 3 | <0.001 | HMGB3 | HMGB3 |
| 12 | Costars family protein ABRACL (ABRA C-terminal-like protein) *(ABRAL)* | -2.63 | 3 | <0.001 | | |
| 13 | Apolipoprotein A-I (B5XBH3) | -2.63 | 15 | <0.001 | APOA1 | APOA1 |
| 14 | Heat shock cognate 70 kDa protein *(B5DFX7)* | -2.50 | 9 | <0.001 | HSP70 | HSPA8 |
| 15 | Stathmin *(B5X953)* | -2.41 | 3 | 0.001 | STMN1 | STMN1 |
| 16 | Proliferating cell nuclear antigen *(B9EMQ6)* | -2.34 | 6 | <0.001 | PCNA | PCNA |
| 17 | Translationally-controlled tumor like protein *(B5XAC1)* | -2.34 | 7 | <0.001 | TCTP | TPT1 |
| 18 | Guanidinoacetate N-methyltransferase *(B5DGB5)* | -2.33 | 5 | <0.001 | GAMT | GAMT |
| 19 | Lipase *(B5X16)* | -2.30 | 7 | 0.002 | LICH | LIPA |
| 20 | NDRG1 *(B5X292)* | -2.24 | 3 | 0.008 | NDRG1 | NDRG1 |
| 21 | Proactivator polypeptide *(B5X4D6)* | -2.20 | 7 | <0.001 | SAP | PSAP |
| 22 | Peroxisomal trans-2-enoyl-CoA reductase *(B5XAK8)* | -2.16 | 7 | <0.001 | PECR | PECR |
| 23 | Elongation factor-1 delta-1 *(B5DGP8)* | -2.13 | 5 | <0.001 | | |
| 24 | Lupus La protein homolog B *(C0HAU7)* | -2.09 | 8 | <0.001 | LAB | |
| 25 | Adenosine kinase a *(B5DGF0)* | -2.08 | 18 | <0.001 | | |
| 26 | Plasminogen activator inhibitor 1 RNA-binding protein *(B5X326)* | -2.03 | 4 | <0.001 | PAIRB | SERBP1 |
| 27 | Beta-carotene oxygenase 2 like *(K8DW80)* | -2.02 | 6 | <0.001 | bco2 | BCO2 |
| 28 | Apolipoprotein B (Fragment) *(Q91480)* | 2.05 | 8 | 0.016 | | APOB |
| 29 | UDP-glucuronosyltransferase (EC 2.4.1.17) *(B5X180)* | 2.16 | 4 | 0.014 | UD2A2 | UGT2A1 |
| 30 | 15-hydroxyprostaglandin dehydrogenase *(B9EPG3)* | 2.19 | 11 | <0.001 | PGDH | HPGD |
| 31 | Phenazine biosynthesis-like domain-containing protein 2 *(C0H855)* | 2.33 | 11 | <0.001 | PBLD2 | PBLD |
| 32 | Metallothionein B (MT-B) *(MTB)* | 2.33 | 2 | 0.004 | mtb | |
| 33 | Serpin H1 *(B5X1Q5)* | 3.07 | 4 | 0.004 | SERPH | SERPINH1 |
| 34 | Ferritin *(C0H793)* | 3.16 | 8 | <0.001 | FRIM | FTL |
| 35 | Digestive cysteine proteinase 2 (B5X4D9) | 3.63 | 7 | <0.001 | CYSP2 | |
| 36 | Canopy homolog 2 *(B5XA82)* | 4.41 | 5 | <0.001 | CNPY2 | CNPY2 |
| 37 | Erythrocyte band 7 integral membrane protein *(B5X1V0)* | 5.01 | 6 | 0.005 | STOM | STOM |

False discovery rate adjustment using Benjamini Hochberg correction. Reported proteins with values < 0.05

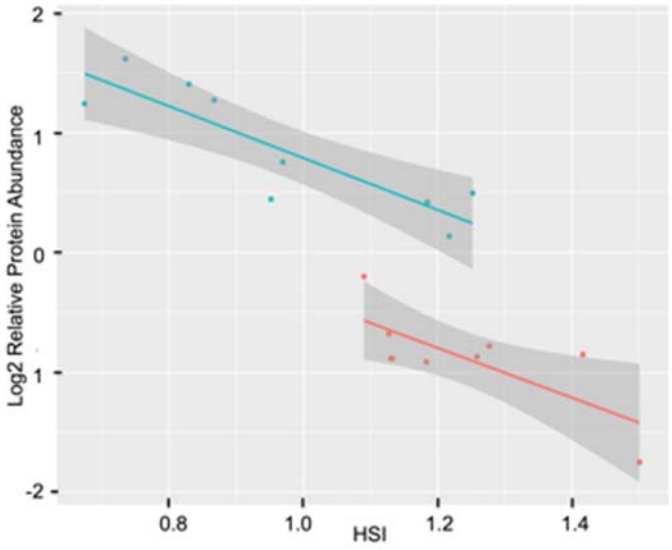


**Figure 5.2. Relative abundance of ferritin (FTL) relative to hepato-somatic index (HSI)**. Color indicates temperature treatment (Blue: 21°C; Red: 15°C). Shaded areas represent 95% confidence intervals. Pearson correlation coefficient across temperature treatments equals to 0.94.

Biological pathway analysis of regulated proteins using IPA software revealed 43 significant canonical pathways. The significance of the association between the data set and the pathway was determined based on the p-value, which determines the probability that the association between the data set genes and the pathway is explained only by chance, and on the ratio value, representing the number of genes from the data found in each pathway over the total number of genes in that pathway. The top five significant canonical pathways included *"EIF2 Signaling"* (-log p-value = 29.2; ratio = 0.17), *"Protein Ubiquitination Pathway" "* (-log p-value = 18.8; ratio = 0.10), *"Regulation of eIF4 and p70S6K Signaling"* (-log p-value = 9.3; ratio = 0.09), *"mTOR Signaling"* (-log p-value = 7.9; ratio = 0.07) and *"Mitochondrial Dysfunction"* (-log p-value = 5.4; ratio = 0.06), while those with the higher ratio included *"Spliceosomal Cycle"* (-log p-value = 1.7; ratio = 0.5), *"Fatty acid β-oxidation"* (-log p-value = 2.8; ratio = 0.33), *"Pentose Phosphate Pathway"* (-log p-value = 4.2; ratio = 0.18), *"β-alanine degradation"* (-log p-value = 2.4; ratio = 0.20), *"Superoxide radicals degradation"* (-log p-value = 2.4; ratio = 0.20), *"Endoplasmic Reticulum Stress Pathway"* (-log p-value = 4.3; ratio = 0.19) and *"Valine Degradation"* (-log p-value = 4.6; ratio = 0.14). The activity pattern of the canonical pathway can be predicted by the activation z-score, a statistical measure of the match between expected relationship direction and observed expression. Predicted activity was found to be increased for *"NRF2-mediated Oxidative Stress Response"* (-log p-value = 4.8; ratio = 0.06; z-score = 0.45). The full list of canonical pathways as determined by temperature-regulated proteins is shown in Supplemental Table 5.4.

Toxicity pathways are canonical pathways that are significantly associated with toxicity lists. These are functional gene groupings based on critical biological processes and key toxicological responses, and describe adaptive, defensive, or reparative responses to xenobiotic insults. The significance of the association is also defined by a p-value and a ratio value. A total of 15 statistically significant toxicity pathways were mined from the temperature-regulated proteins. The five most significant pathways also showed the highest ratios and included *"Fatty acid metabolism"* (-log p-value =24.8; ratio = 0.09), *"Increases Transmembrane Potential of Mitochondria and Mitochondrial Membrane"* (-log p-value = 20.4; ratio = 0.14), *"Mitochondrial Dysfunction"* (-log p-value = 18.9; ratio = 0.06), *"Glutathione Depletion-CYP Induction and Reactive Metabolites"* (-log p-value = 17.6; ratio =0.33) and *"Oxidative Stress"* (-log p-value = 15.3; ratio = 0.11). The components of each of the top five toxicity pathways are shown in Supplemental Table 5.5.

IPA software identified 15 top networks and ranked them by a score that considers the number of focus proteins and the size of the network to approximate the relevance of the network to the original list of focus proteins. Top five networks revealed links with *"RNA Post-Transcriptional Modification, Cellular Assembly and Organization"* (score 52), "*Cell-To-Cell Signaling and Interaction", "Cancer, Cell Death and Survival, Organismal Injury and Abnormalities"* (score 44), *"Protein Synthesis, Gene Expression, Developmental Disorder"* (score 39), *"Protein Trafficking, Molecular Transport, Cellular Compromise"* (score 37), and *"Lipid Metabolism, Small Molecule Biochemistry, Molecular Transport"* (score 35). The components of the top four networks are shown in Figure 5.3.

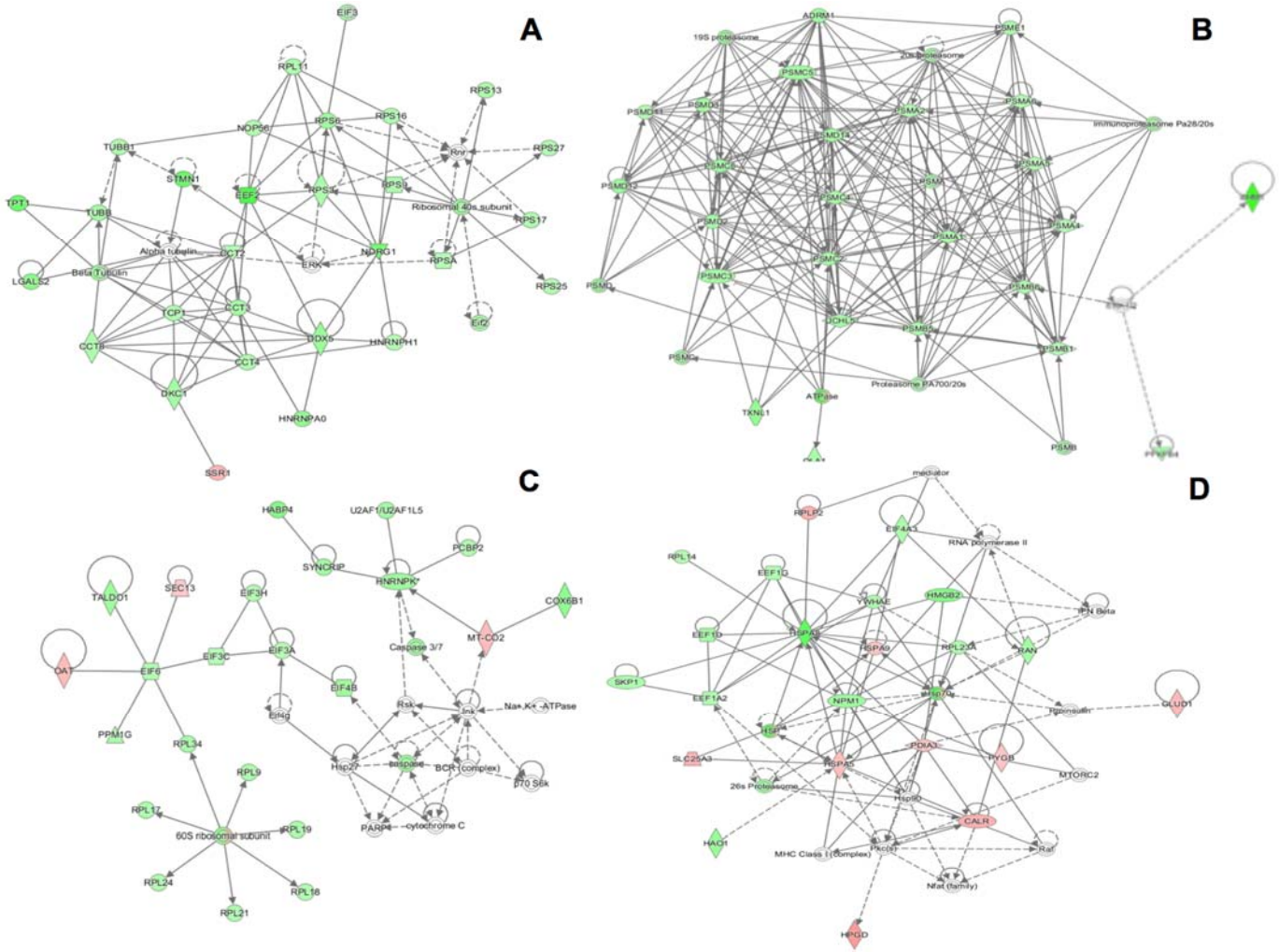


**Figure 5.3. Top four-scoring biological networks for the significantly elevated temperature (21°C) regulated proteins in liver of Atlantic salmon using IPA analysis.** A) RNA Post-Transcriptional Modification, Cellular Assembly and Organization, B) Cell-To-Cell Signaling and Interaction", "Cancer, Cell Death and Survival, Organismal Injury and Abnormalities, C) Protein Synthesis, Gene Expression, Developmental Disorder, and D) Protein Trafficking, Molecular Transport, Cellular Compromise. Nodes are colored according to increased (red) or decreased (green) abundance at elevated temperature.

## 5.5. DISCUSSION

We have used shotgun proteomics analysis to characterize changes in the liver proteome of Atlantic salmon following exposure to chronic elevated temperature (21°C). To our knowledge, this is the first study to provide proteome-level evidence of such response in Atlantic salmon and other aquaculture species. The metabolic role and plasticity of liver tissue in the thermal adaptive process was reflected in the clear separation between temperature treatments by MDS and in the high number of proteins that were regulated under 21°C. The general pattern of down-regulation is in line with the liver transcriptome response of similar size Atlantic salmon upon chronic elevated temperature (Olsvik et al., 2013). The molecular dynamics of adaptation to thermal stress are dependent on tissue type examined, type of stress, and thermal exposure regime (Logan and Buckley, 2015). Specifically in liver tissue, metabolic down-regulation and small magnitude response of stress indicators appears to be associated with chronic exposure to elevated temperature (Olsvik et al., 2013, Mininni et al., 2014), while an up-regulated response and larger fold changes are elicited in response to more acute thermal stresses (Ibarz et al., 2010, Mahanty et al., 2016).

Physiological adjustments towards homeostasis were paralleled by constraints in performance. Metabolic rate increases exponentially as temperature increases and, at any given temperature, the difference between feed intake and metabolic rate will determine the energy available for tissue development and fish growth (Jobling, 1994). Given the similar feed intake between temperature treatments, the lower biometric indices (HSI, k) in the 21°C fish suggested that the energy deficit caused by the increased energy demand was not met by feed intake. The proteomic data described here correlate with the biometric data and provide new insights into the main biological processes involved in the liver adaptive response of Atlantic salmon to chronic elevated temperature.

### 5.5.1. Protein synthesis and degradation

Protein synthesis is a major energy consuming process that can account for up to 42% of total energy expenditure in fish. Liver tissue displays the highest rates of protein synthesis (Carter and Houlihan, 2001), and so is considered a highly sensitive physiological indicator of the impact of elevated temperature (Morgan et al., 2001, Katersky and Carter, 2010). Consistent with previous liver transcriptional data in Atlantic salmon (Olsvik et al., 2013), our proteomic data demonstrated that chronic elevated sub-lethal temperature suppresses protein synthesis as a compensatory mechanism to the induced energy deficit. This was mediated in both studies through two independent mechanisms: eukaryotic initiation factor 2 (EIF2) signaling and the mechanistic target of rapamycin (mTOR) signaling pathway. Both regulatory functions respond to energy status and stress within the cell and are performed by serine/threonine kinases and phosphatases via phosphorylation-dephosphorylation of EIF2α (Stolboushkina and Garber, 2011) and downstream effectors such as eiF4 and EEF2 (Showkat et al., 2014). Accordingly, reduced expression levels of ribosomal proteins (25 proteins), translation

initiation (8 proteins) and elongation factors (5 proteins), including EIF2, eiF4 and EEF2 subunits, were accompanied by reduced serine/threonine phosphatase activity (PP1CC and PPM1G).

Repression of the translational machinery was also reflected upstream in the inhibition of pre-translational regulatory mechanisms, as well as down-stream in the inhibition of cytoplasmic chaperones that assist in the folding and trafficking of nascent proteins. Down-regulation of heterogeneous nuclear ribonucleoproteins (HNRNPK, HNRNPH1, HNRNPAB, HNRNPA0, SYNCRIP, PCBP2), responsible for packing and stabilizing freshly transcribed pre-mRNA (Geuens et al., 2016), mirrored a reduced demand mRNA export out of the nucleus to translation active sites in the cytoplasm. Noteworthy was the down-regulation of chromatin regulators, including high mobility group proteins (HMGB2 and HMGB3), the intracellular hyaluran-binding protein (HABP4) and nucleophosmin (NMP1). Chromatin remodelling appears to be a critical process in compensating for elevated temperature effects (Portner et al., 2012), as also suggested in the liver transcriptome analysis of heat-stressed eurythermal annual killsfish (*Austrofundulus limnaeus*) (Podrabsky and Somero, 2004). In cytoplasm, reduced chaperone demand was indicated by the 2.5-fold down-regulation of the heat shock cognate 71kDa protein (HSPA8), which contributes to the overall cytoplasmic folding by binding approximately 20% of the new translated proteins (Stricher et al., 2013). Specifically upon heat stress, the trafficking activity of HSPA8 was inhibited and reestablished after recovery (Kodiha et al., 2005). The chaperonin-containing TCP1 complex (TCP1, CCT2, CCT3, CCT4, CCT8) was also down-regulated. While the TCP1 complex is involved in the folding of approximately 1% of newly synthesized proteins (Dekker et al., 2008), these mostly include microtubule proteins, which were also regulated as a consequence of thermal stress, and are discussed below in the context of cytoskeletal integrity.

An important finding of this study was the inhibitory effect of chronic elevated temperature on protein degradation via the ATP-dependent ubiquitin-proteasome pathway. Proteins involved in ubiquitin conjugation (UBE2V1, UBE2D2, UBQLN4), ubiquitin ligase activation (SKP, TCEB2) and de-ubiquitination (UCLH5, ADRM1), and in the subsequent proteasome degradation (5 PSMCs, 5 PSMDs, 5 PSMAs, 1 PSME), were down-regulated. This was additionally paralleled by the down-regulation of cathepsins (CTSD, CATM) involved in lysosomal protein degradation pathway. Specifically, CTSD has been previously measured as an indicator of protein degradation in response to temperature stress in fish (Mommsen, 2004, Carter et al., 2008). Signs of suppressed protein degradation were however not detected in the liver transcriptome of similar size Atlantic salmon exposed to chronic elevated temperature, in that case at 19°C (Olsvik et al., 2013). This observation corroborates the impact that the extra energy deficit generated by an additional 2°C has on protein metabolism; under restricted feeding, such difference in rainbow trout reduced the liver rates of protein synthesis and degradation by ~35-55% (Morgan et al., 1999). Reduced protein degradation is therefore a compensatory mechanism that follows protein synthesis inhibition in order to maintain growth and conserve energy under a temperature-induced energy deficit not compensated by feed intake. While this

idea is well established at temperatures within the upper critical range (Reid et al., 1997), it also seems to apply to the chronic exposure to temperatures considered to be within the range of optimal thermal tolerance, albeit at the upper limit.

### 5.5.2. Energy and lipid metabolism

The liver is a central hub for the storage and conversion of high-energy substrates. Under optimal temperature and feeding conditions, fish use amino acids rather than glucose as preferential energy source, while the contribution of fatty acid oxidation to energy production is correlated to dietary lipid levels (Médale and Guillaume, 2001). At elevated temperature, energy metabolism is remodeled to compensate for the deficiencies derived from increased metabolic rate (Windisch et al., 2014). Consistent with the trend observed in the plasma metabolome of Atlantic salmon (Kullgren et al., 2013), our proteomic data suggested an increased dependence on amino acids rather than glucose and fatty acids for energy production upon chronic elevated temperature. This was reflected in the up-regulation of mitochondrial enzymes involved in the degradation of valine (HIBADH, HIBC), tryptophan (AFMID, GCDH) and leucine (MCCC1, MCCC2, IVD) towards the formation of citric acid (TCA) cycle intermediates, and supported by the increased expression of TCA enzymes such as glutamate dehydrogenase (GLUD1) and aspartate aminotransferase (GOT2). The apparent increased TCA flux from amino acid catabolism was paralleled by signs of reduced glucose availability, including up-regulation of glycogen catabolic enzymes (PYGB, MTAP), down-regulation of several proteins involved in the pentose phosphate pathway (PGD, RPE, TKT, TALDO1), and the 2.7-fold down-regulation of pyruvate kinase (KPYK). Mitochondrial β-oxidation of fatty acids also showed signs of suppression (ACAD11, ECI2). This may be linked to the fact that, under unrestricted feeding, TCA dependence on fatty acid oxidation diminishes with increasing exposure time to elevated temperature (Kullgren et al., 2013).

Increasing temperature increases the fluidity of cell membrane, leading to alterations in lipid metabolism to maintain and stabilize fluidity (Los and Murata, 2004, Miller et al., 2006). In this study, several proteins associated with fatty acid metabolism and lipid transport were down-regulated at elevated temperature and linked to mechanisms involved in modulating membrane fluidity. The peroxisomal acyl-coenzyme A oxidase 3 (ACOX3), the rate-limiting enzyme in the oxidative breakdown of methyl-branched fatty acids (Hunt et al., 2012), showed the largest fold change (× 5.4). Since methyl-branched fatty acids have been suggested to enhance the fluidity of the membrane lipid bilayers over different environmental conditions (Poger et al., 2014), ACOX3 down-regulation may be part of a mechanism to increase membrane fluidity at elevated temperature. Membrane fluidity is stabilized by the incorporation of cholesterol into lipid bilayers, with increased cholesterol levels associated to increased temperatures (Robertson and Hazel, 1997). An important mechanism in cholesterol homeostasis is the reverse cholesterol transport (RCT), whereby cholesterol is transported in high-density lipoproteins (HDL) proteins from peripheral tissues back to the liver to be further

eliminated in the bile (Ghosh, 2012). Elevated temperature appeared to inhibit RCT, as reflected by the parallel down-regulation of apolipoprotein A1 (APOA1) (× 2.6), the principal component of HDL, and lipase (LIPA) (× 2.3), involved in HDL uptake and a well-known rate-limiting enzyme in RCT (Ghosh, 2012). Subsequent signs of altered cholesterol metabolism included down-regulation of epididymal secretory protein E1 (NCP2) (× 1.7), an intracellular cholesterol transporter that regulates cholesterol biliary secretion, and of 3-oxo-5-beta-steroid 4-dehydrogenase (AKR1D1) (× 2.0), involved in cholesterol breakdown towards the synthesis of bile acids. These observations emphasize the importance of cholesterol in the acclimatory response to elevated temperature (Podrabsky and Somero, 2004), and further suggest suppression of reverse transport and catabolism of cholesterol as a compensatory response to the increased peripheral retention induced by elevated temperature.

### 5.5.3. Cytoskeletal integrity

Stress-induced perturbations of the transcription and translational mechanisms can block cell growth and proliferation via alteration of the cytoskeletal integrity (Kim and Coulombe, 2010). Thus, the microtubule network of α- and β-tubulins forming the cell cytoskeleton is recognized to play a role in maintenance of cell homeostasis and execution of a variety of cell stress responses (Parker et al., 2014). In line with a previous observation in liver transcriptome of Antarctic fish (Windisch et al., 2014), the liver proteome of Atlantic salmon reflected suppression of the microtubule dynamics in response to chronic elevated temperature. Down-regulation of tubulins (TUBB, TUBB1) was paralleled by down-regulation of the chaperonin-containing TCP1 complex (TCP1, CCT2, CCT3, CCT4, CCT8), which is particularly implicated in the folding and assembly of tubulins in an ATP-dependent manner (Dekker et al., 2008). Notably, microtubule-stabilizing proteins were also down-regulated, including tubulin-folding factor (TBCB) (Szymanski, 2002), translationally-controlled tumor protein (TPT1) (Chan et al., 2012), N-myc downstream regulated gene 1 (NDRG1) (Melotte et al., 2010), and stathmin (STMN1) (Rubin and Atweh, 2004). We highlight the larger than two-fold changes in TPT1 and NDRG1, established hallmarks and mediators of cell proliferation through microtubule stabilization. A novel outcome of the heat-induced cytoskeleton remodeling was the observed 4.4-fold down-regulation of the canopy homologue 2 (CNPY2). CNPY2 is a transmembrane protein that regulates myosin regulatory light (MRLC) protein levels, a protein that links cytoskeleton to membrane proteins and stimulates cell growth (Hatta et al., 2014). Reduced cytoskeletal integrity at elevated temperature has been attributed to a the maintenance of a less dense subcellular structure resulting from alterations in cytosol solubility and viscosity (Windisch et al., 2014), or to an increase in the pool of soluble tubulin as a result of oxidative stress-induced microtubule depolymerization (Parker et al., 2014). In contrast to our results, acute heat stress induced up-regulation of genes encoding cytoskeleton components in gill tissue of Pacific salmon (Tomalty et al., 2015). This difference between studies corroborates the tissue- and exposure regime- specificity of the heat stress response across different protein groups and biological processes (Logan and Buckley, 2015).

### 5.5.4. Oxidative stress and endoplasmic reticulum (ER) stress

Exposing ectotherms to elevated temperatures challenges the cellular redox balance and lead to increased production of reactive oxygen species (ROS) (Tomanek, 2015). Mitochondria are well-known as major sites of ROS production and consumption and are implicated as the main source of thermally-induced oxidative stress (Banh et al., 2016). ROS imbalance occurs due to the uncoupling of mitochondrial respiration; a proton gradient generated by complex I, III and IV, but not coupled with consumption by complex V, will leak, consequently increasing the mitochondrial membrane potential and ROS formation (Rousset et al., 2004, Srinivasan and Avadhani, 2012). This mechanism, previously proposed as cause of ROS formation in polar and temperate fish upon long-term warm acclimation (Mark et al., 2006), was here corroborated in Atlantic salmon; the predicted mitochondrial dysfunction, encompassing imbalances in the electron transport complexes IV (COXAI2, COX6B1, COX7A2L, MT-CO2) and V (ATPL5L), was paralleled by the predicted increased transmembrane potential of mitochondria. The ROS-induced cellular antioxidant response was mediated via nuclear factor erythroid 2-related factor 2 (Nrf2) through the regulation of different mechanisms (Ma, 2013). These included induction of catabolism of the ROS superoxide through mitochondrial superoxide dismutase (SOD2) and the peroxiredoxin system, and metal chelation by ferritin (FTL). Unlike SOD2 up-regulation, peroxiredoxins exhibited opposite patterns of regulation. This expression pattern has been observed in different oxidative stress related disorders (Pagano et al., 2014) and attributed to the increased exposure to the superoxide derivative hydrogen peroxide (Rhee et al., 2005, Cox et al., 2008). Specifically, the observed co-up-regulation of PRDX3 and PRDX4 has been proposed as a biomarker of antioxidant response and oxidative damage in cancer (Basu et al., 2011). The 3-fold up-regulation of FTL was consistent with the increased transcript level and protein abundance in *Channa* liver upon chronic elevated temperature (Mahanty et al., 2016). Iron interacts with hydrogen peroxide via the Fenton reaction leading to the production of hydroxyl radical ROS, a very reactive initiator of lipid peroxidation (Chen et al., 2012). As FTL maintains iron homeostasis, overexpression suggested the cell's attempt to prevent Fenton type reactions and ROS accumulation (Orino et al., 2001). The Nrf2-mediated antioxidant response was further supported by the 2.3-fold up-regulation of metallothionein B (mtb), a cysteine-rich metal binding protein involved in metal homeostasis and ROS scavenging (Ruttkay-Nedecky et al., 2013). Induction of the ROS scavenging system was also paralleled by the up-regulation of cytosolic epoxide hydrolases (EPHX1, EPHX2) and aldo-keto reductases (AKR7A2, AKR1B1), respectively involved in the detoxification of fatty acid epoxides and aldehydes (Fretland and Omiecinski, 2000, Penning, 2015), and suggesting some of degree of lipid peroxidation in 21°C livers as previously detected in heat-stressed fish (Kaur et al., 2005, Lushchak and Bagnyukova, 2006). A contribution of our study towards biomarker discovery was the significant and negative correlation between FTL expression and HSI across fish of both 15°C and 21°C groups. FTL is used as a reliable blood marker of liver disorders in human medicine (Adams, 2008), and it might be interesting to further

explore the FTL expression levels in blood as a surrogate and less invasive measurement of liver condition and oxidative stress in fish.

Oxidative stress is a condition of imbalance between the formation of ROS and the biological system's ability to detoxify the reactive intermediates, thus dysfunction of the antioxidant and detoxifying activity is also reflective of oxidative damage (Banh et al., 2016). This concept is consistent with previous thermal stress studies in fish examining varying exposure regimes (Logan and Buckley, 2015, Tomanek, 2015) and was supported here by the down-regulation of catalase (CAT), glutathione transferases (GSTs: GSTT1 and GSTP1), and betaine-homocysteine S-methyltransferase 1 (BHMT) (×3.4) at elevated temperature. CAT is a mitochondrial and peroxisomal consumer of hydrogen peroxide, though down-regulation in fish has been attributed to its sensitivity to the fluctuation of superoxide radicals (Wilhelm Filho, 1996). Glutathione plays a central role in the cellular defence against lipid peroxidation (Ayala et al., 2014). While GSTs catalyse the metabolism of lipid peroxides by conjugation with glutathione and NADPH (Xiao et al., 2009), BHMT maintains steady levels of the glutathione precursor s-adenosyl-methionine (SAM) (i.e. SAM is the amino acid methionine bound to an ATP molecule) (Obeid, 2013). Co-down-regulation of GSTs and BHMT was thus indicative of glutathione depletion and reduced hepatoprotection, as previously detected and associated to increased lipid peroxidation in liver of fish exposed to thermal stress (Kaur et al., 2005, Lushchak and Bagnyukova, 2006, Ibarz et al., 2010). These results collectively target the methionine cycle as a possible via to mitigate the impaired enzymatic hepato-protection associated to thermal stress. Enhanced protection was recently suggested in seabream fed a winter-specific diet supplemented with methionine (Richard et al., 2016). A comparison of our proteomic data with the liver transcriptome of similar size Atlantic salmon upon chronic exposure to 19°C (Olsvik et al., 2013) implicates variation in the oxidative stress response that attributes to temperature-specific-effects and the consequent ROS production (Logan and Buckley, 2015, Banh et al., 2016). This highlights the risk of drawing general conclusions and also the importance of other experimental factors such as dietary formulation (e.g. antioxidant supplementation) and methodology used (e.g. proteomics vs. transcriptomics, pooling vs. individual-based analysis) in identifying thermal stressors across studies.

Accumulating evidence corroborates the intrinsic link between cellular oxidative stress and ER stress (Malhotra and Kaufman, 2007). One proposed mechanism for this interrelation is that mitochondrial ROS formation promotes the calcium release from the ER, leading to the accumulation of unfolded proteins in the ER lumen and further contributing to ROS formation due to the excessive calcium influx into the mitochondria (Cribb et al., 2005). The ER unfolded protein response (UPR) is subsequently activated and mediated through the induction of molecular chaperones to restore proteostasis and avoid apoptosis. At elevated temperature, activation of the UPR response was featured by the up-regulation of following chaperone proteins: calreticulin (CALR), reflecting increased calcium release from ER but also increased protein folding (Cribb et al., 2005); disulfide isomerases (PDIA3, PDIA6), reflecting disulfide bond rearrangement and increased protein folding (Perri et al., 2015); and

78 kDa glucose-regulated protein (HSPA5, also termed GRP78), reflecting increased retention and degradation of misfolded proteins (Wang et al., 2009). Additionally, ER glycosyltransferases (UGT2A1, RPN1) were up-regulated, providing further evidence for the increased folding activities via increased N-glycosylation (Gerlach et al., 2012). Our data confirm previous detections of ER stress and UPR in liver of thermal-stressed fish (Ibarz et al., 2010, Mininni et al., 2014, Mahanty et al., 2016, Richard et al., 2016) and demonstrate its specific importance in the adaptive response to chronic elevated temperature. Another indicator of the crosstalk between cellular oxidative stress and ER stress was the 3-fold up-regulation of serpin H1 (SERPINH1). This protein is an ER chaperone exclusive for the binding and stabilization of collagen, whose synthesis can be triggered by stress induced-lipid peroxidation (Wu and Cederbaum, 2009, Cichoż-Lach and Michalak, 2014). In line with our study, increased transcript levels of SERPINH1 were found in gill tissue of Pacific salmon under different heat exposure regimes (Jeffries et al., 2012, Jeffries et al., 2014, Tomalty et al., 2015).

### 5.5.5. Conclusion

This study increases our understanding of the liver molecular mechanisms in Atlantic salmon that are important for coping with chronic elevated temperature, which is of increasing importance in temperate production areas such as Tasmania in Southern Australia. Suppression of protein synthesis and degradation mechanism appears to be the main energy saving mechanism for the increased metabolic demand, which is also reflected in an increased dependence of amino acid catabolism towards energy production. Other chronic heat-stress related mechanisms included the reverse transport and catabolism of cholesterol, cytoskeletal dynamics, the Nrf2-mediated antioxidant response and the endoplasmic UPR. Many of the proteins regulated here are common to the thermal stress response across fish species and tissues. These included protein groups (TCP1 complex proteins, high mobility group proteins, disulfide isomerases, peroxiredoxins and glutathione transferases) and individual proteins (CTSD, SOD2, FTL, BHMT, HSPA5, CALR and SERPINH1). Other stressors, particularly several cytoskeletal-related proteins (STMN1, TPT1, NDRG1 and CNPY2) were for first time here reported in response to thermal stress. Finally, opportunities for new research towards the development of salmon feed formulations specific to the summer period are raised here. Further understanding of methionine supplementation to improve the liver detoxifying capacity, or the evaluation of ingredients or additives to compensate the energy deficit and to spare amino acid degradation towards energy production are warranted.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD004985.

# CHAPTER 6

# General discussion and concluding remarks

## 6.1. GENERAL DISCUSSION

Improved growth and production efficiency, product quality and sustainability are all fundamental to the ongoing success of the Atlantic salmon aquaculture industry. The research presented in this thesis has enhanced our physiological understanding of how current production strategies and environmental factors, in particular those relevant to Tasmanian industry growing conditions, can affect phenotypic traits in Atlantic salmon. The following discussion relates to the major findings of this thesis and to their implications, highlights areas for further research in salmon aquaculture and further potential applications of proteomics in aquaculture research.

### 6.1.1. Triploidy

Triploid salmon has been traditionally considered inferior to the diploid counterpart, and this view was based on lower growth and the higher incidence of skeletal anomalies observed early in the development of the industry (Sutterlin et al., 1984, Carter et al., 1994, Benfey, 2001). Triploid research since then has benefitted the industry through the development of specific husbandry and nutritional strategies (Benfey, 2015). In Chapter 3, using optimized growing conditions, only subtle changes were observed between diploid and triploid fish at the phenotypic level. The lack of detectable differences at the proteome level was consistent with these data, and supports current industry standards in producing high quality smolts (Chapter 3). This finding also importantly corroborates the idea that triploid inferiority is rather a consequence of sub-optimal conditions such as feed deprivation (Cleveland and Weber, 2013) or disease (Ching et al., 2010). Regulatory processes exhibit a dosage-dependent behavior whereby the phenotypic characteristics derived from the expression of specific genes may be cancelled by the expression of other genes producing an inverse dosage effect (Birchler et al., 2001). In triploids, sub-optimal or stressing conditions can disrupt this dosage compensation mechanism for several genes (e.g. immune- and protein metabolism-related), with the increased gene dosage adversely affecting gene expression and phenotypic traits. A general question remaining relates to the level of stress required to disrupt the dosage compensation mechanism in triploid fish. This aspect is relevant to other key aspects of salmon production, including the effect of an extensive regulating factor such as elevated water temperature (Chapter 5), and a non-stressful but metabolically challenging factor such as dietary oil manipulation (Chapter 4). Further assessment of the interaction between these and other factors and in addition triploidy will also contribute to refining the development of triploid-specific nutritional and husbandry strategies.

The high incidence of skeletal deformities is still the main concern in farmed triploid salmon (Benfey, 2015). Strong evidence suggests that skeletal anomalies result primarily from the triploid induction process (Imsland et al., 2014, Weber et al., 2014). Further studies using proteomics could identify molecular changes in cartilage underpinning heat shock pressure induction in early freshwater stages and thereby contribute to the fine-tuning of the current triploidization procedures. Lower jaw

deformities in triploid salmon represent a major problem in Tasmanian aquaculture, affecting up to 30% of the triploid population (Amoroso et al., 2016b). Further studies using shotgun proteomics would complement recent analysis of gene expression changes (Amoroso, unpublished data) to explore and identify mechanisms in cartilage and bone formation that are driving the development of jaw deformities in late freshwater and seawater stages. An advantage of using solubility-based sequential protein extraction (Chapter 2) would be to identify differences not just in protein abundance but also their relative 'extractability' (Wilson et al., 2010), which could reveal defects in proper formation of the cartilage extracellular matrix underlying the development of skeletal deformities. Given the well-demonstrated family effect on the incidence of skeletal anomalies (Taylor et al., 2011, Taylor et al., 2013), the use of full siblings is critical to avoid genetic heterogeneity and to provide a clear-cut approach.

### 6.1.2. Use of a model oil blend with high DHA and ALA content

Novel oils extracted from transgenic seeds are presently produced in limited quantities. Chapter 4 highlighted the usefulness of formulating a model or mimic oil to assess the use of such oils in fish trials, to understand more precisely their biological utilization, primarily retention or oxidation, and ultimately to drive the formulation of sustainable aquafeeds in using these novel oils. A potential criticism of the model oil approach is that the molecular species of triacylglycerols (TAG) and the distribution of fatty acids on the TAG backbone (i.e. termed the regiospecificity) might differ between the model oil and the "real" oil; this in turn may affect the ability of the model oil to accurately reflect the biological utilization of fatty acids. However, it must be emphasized that evidence of such differences is not presently available and it is likely to be minimal, since the metabolic fates of fatty acids seem to be primarily concentration-dependent and the model oil predictions match observations across other studies (e.g. EPA is preferentially oxidized over DHA, ALA is bioconverted).

The blended oil approach currently practiced by the aquafeed industry in Australia generally uses a high proportion of poultry oil blended with lower quantities of fish oil. This maintains the content of omega-3 long-chain ($\geq C_{20}$) polyunsaturated fatty acids (n-3 LC-PUFA) in the salmon fillet (Nichols et al., 2014). The availability of novel DHA-containing oilseeds, such as that from transgenic *Camelina* and/or canola (Petrie et al., 2012, Petrie et al., 2013), provides the feed formulator with the additional option of increasing the docosahexaenoic acid (DHA) content of the blend oil and ultimately to improve the n-3 LC-PUFA content of the salmon fillet. If this is used as the only oil source in feed, approximately 80% of the n-3 LC-PUFA content achieved with a traditional fish oil-based diet can be potentially reached. A major variable that determines the price of oils is the relative levels of DHA and eicosapentaenoic acid (EPA), with both having the same cost per unit (Grupo Dibaq, personal communication). Chapter 4 corroborates that the utilization of DHA and EPA by salmon is, however, not equally efficient, and DHA is preferentially deposited and EPA is more extensively oxidized (Stubhaug et al., 2007, Codabaccus et al., 2012). Therefore, on the same absolute content of n-3 LC-

PUFA in oil, DHA is economically more efficient than EPA, and this is the major advantage that DHA-containing oils offer in relation to EPA- (Betancor et al., 2015b) and EPA-DHA-containing oils (Betancor et al., 2016) for use in aquafeeds. From the consumer perspective, the advantage of using DHA-containing oils over EPA-containing oils in aquafeeds is the higher n-3:n-6 ratio (10:1 vs. 1:1, respectively), which positively impacts the potential health benefits derived from eating salmon (Simopoulos, 2008, Williams et al., 2011, Simopoulos, 2016).

Chapter 4 also demonstrated the usefulness of proteomics in complementing the formulation of a model oil blend to understand diet-induced physiological changes in fish towards optimizing the use of novel oils in aquafeeds. Several proteins previously associated with oxidative stress and xenobiotic metabolism were altered (GSTT1, CP2M1, ALDH2, UD2A2), providing evidence for an oxidative stress response, potentially induced by peroxidation of lipid in the liver of fish fed the model oil. This observation indicates that the use of DHA-containing *Camelina* or canola oil at high inclusion rates may require particular attention to the increased risk of oxidative damage and lipid peroxidation, presumably generated by the unusual combination of high DHA, high ALA and consequently high total PUFA. Indeed, new oilseed-derived ALA-rich and DHA-containing oils developed by CSIRO (Peter Nichols, unpublished data) include a range of antioxidants at levels likely to mitigate any possible risks. This aspect also requires further consideration in the context of summer conditions in Tasmania, where salmon vulnerability to oxidative stress could be exacerbated by elevated water temperature (Chapter 5).

An important attribute of the DHA-containing oil from *Camelina* and/or canola is the high α-linolenic acid (ALA) content (Petrie et al., 2012, Petrie et al., 2013). Increasing the level of ALA in commercial diets has been recommended to promote the growth of beneficial intestinal bacteria (Ringø et al., 2016), but is also associated with accumulation of lipid droplets in the enterocytes and cell damage (Olsen et al., 1999b, Olsen et al., 2000). Determining adequate inclusion rates of transgenic *Camelina* and/or canola oil also requires understanding of the benefits or detriments to intestinal health. Further analysis, for example using proteomics, may provide insights on the possible effects on intestinal barrier integrity and epithelial-cell metabolism.

### 6.1.3. Heat stress

The use of proteomics has refined our understanding on how hepatic metabolism in Atlantic salmon is remodeled upon exposure to chronic elevated temperature (Chapter 5). The metabolic rate of fish increases as water temperature increases, resulting in an energy deficit if the increased energy demand is not compensated by increased feed intake (Jobling, 1994). A major finding of the current research was that both protein synthesis and protein degradation pathways were suppressed to compensate for the energy deficit following chronic exposure to 21°C. Protein synthesis and protein degradation are metabolically expensive and therefore impact on energy available for growth (Carter and Houlihan, 2001). The increased energy demand was met by an increased dependence on catabolism

of amino acids (valine, tryptophan, leucine) and reduced dependence on glucose and fatty acid metabolism. Remarkably, this pattern of energy remodeling matched observations in the plasma metabolome of Atlantic salmon (Kullgren et al., 2013), and thus it should be considered in the further development of summer feeds. Previously assessed indicators of protein synthesis (EEF2, heterogeneous nuclear ribonucleoproteins, high mobility group proteins) and degradation (20s proteasome subunits, 26s proteasome subunits) in fish were also confirmed here.

Another observed major response, common to other thermal stress studies in fish (Logan and Buckley, 2015), was oxidative stress. A number of oxidative stress candidate biomarkers have been previously suggested across various studies, and some of them were also regulated in this study (SOD2, FTL, GSTT1, BHMT, HSPA5, CALR, SERPINH1). Furthermore, the sensitivity of cytoskeletal dynamics to thermal stress (Windisch et al., 2014, Tomalty et al., 2015), and more specifically to oxidative stress induced-alterations in cytosol solubility (Parker et al., 2014), was corroborated. Larger fold changes (> 2) in several proteins related to cytoskeletal remodeling (STMN1, TPT1, NDRG1, CNPY2) were for first time reported here and their potential as fish stress markers should be further validated.

Once again, this thesis demonstrates the usefulness of proteomics towards optimizing feed formulation, and specifically for feeds targeting summer growing conditions. The use of additional antioxidant supplementation to improve the antioxidant balance and to enable animals to cope with oxidative stress at elevated temperatures is practiced by aquafeed companies (Skretting, 2016). Despite the recent trend to use higher levels of lipids to partially spare protein and help to reduce feed costs (Refstie et al., 2001), the increased amino acid requirement upon heat stress suggests the need of supplementation (Carter et al., 2008). Feed additives such as glycerol (Silva et al., 2012) and sodium butyrate (Robles et al., 2013) also require further consideration as cost-effective energy sources that potentially may enable sparing in amino acid catabolism. Given the sensitivity of the liver proteome to reflect protein and energy metabolism remodeling and the oxidative stress response, proteomics can improve our understanding on how dietary factors can partially mitigate the effects of heat stress. The aforementioned candidate biomarkers should be targeted in further studies to verify the extent to which they can provide insight as measures of stress. One question for consideration is about the capability of shotgun proteomics to predict changes in the requirements of specific amino acids. If effective, this could be useful as a preliminary and exploratory approach to nutritional studies aiming to correct amino acid imbalances.

### 6.1.4. Proteomics in aquaculture research

In the last decade, proteomics has been increasingly used in fish biology research (Rodrigues et al., 2012, Zhou et al., 2012). The usefulness of this technique lies in its sensitivity to detect signs of metabolic shifts or physiological stress when these might not be evident macroscopically or through other targeted means (Silva, 2013). An important goal of this thesis has related to the optimization of

proteomics methodologies to enhance such sensitivity. Protein identification rates reported here were generally higher than in most previous aquaculture proteomic studies, this being attributed to the use of the label-free shotgun approach rather than the traditional gel-based approach. We are at the transition period in aquaculture research where gel-based approaches are shifted towards shotgun approaches, generally allowing for higher identification rates and increased power to identify affected biological pathways. Increased genomic resources, as recently for Atlantic salmon (Lien et al. 2016), more complete protein databases (e.g. PRIDE), and the continuous improvement of the bioinformatics tools used for gene ontology (GO) enrichment (e.g. DAVID, IPA), are crucial for the biological interpretation of the experiments with non-model species. The application of solubility-based sequential protein extraction as a strategy to reduce sample complexity is clearly beneficial in the analysis of structurally complex biological samples. This method provided deeper proteome coverage in larvae samples and was also successfully applied to proteomic analysis of muscle tissue (Chapter 3). As a method to improve protein detection, sequential extraction is one effective approach to compensate for the still limited sequence databases available for "non-model" species.

Fractionation strategies also offer potential for the proteomic analysis of less structurally complex protein matrices, such as liver tissue, in order to address aquaculture topics of current interest. An example is dietary oil manipulation studies (Chapter 4), and the interest to understand how dietary changes influence liver metabolic pathways that determine fatty acid deposition. While mitochondrial and peroxisomal enzymes with a β-oxidative role were identified in all liver proteomic datasets (Chapters 3 – 5), ER and microsomal elongases and desaturases were not detected, most likely due to low expression levels. Therefore, more detailed investigation of elongation and desaturation activities could be addressed using subcellular fractionation to enrich ER proteins.

Considering the liver response to heat stress (Chapter 5), as compared with dietary oil manipulation (Chapter 4), the greater magnitude in protein abundance changes measured was consistent with the idea that stress factors induce broader and deeper changes than dietary factors (Silva, 2013). However, an important further consideration is the impact of experimental design and the relative ability of pooling (Chapter 4) versus individual based analysis (Chapter 5) to estimate small fold changes. Pooling reduces the variance, which is inversely related to the accuracy to determine abundance and the ability to identify differentially expressed proteins, but also reduces the statistical power, which is required to pick up statistically significant, small and consistent changes (Kendziorski et al., 2005). Since the small magnitude response of the liver proteome to dietary oil manipulation was consistent with the intestine proteome (Morais et al., 2012) and the liver transcriptome (Betancor et al., 2015b), pooling by tank is discouraged in this sort of nutritional studies. Increased statistical power and optimized detection of differences in expression levels can be achieved by performing individual-based analysis, with proper specification of random factors (e.g. tank effect), or by having a higher number of pools per treatment (i.e. based on results from Chapter 4, at least over five or biological replicates). If budget limitations only allows for the analysis of a low number of samples (e.g. less than five), the

advantages of pooling can only be gained by pooling a large number of subjects (Kendziorski et al., 2005).

Statistical tools are essential for achieving good statistical power and preventing false conclusions. User-friendly bioinformatics software such as Perseus does not allow for the incorporation of experimental factors (e.g., tank and system effects) into the statistical analysis of protein expression data, which negatively affects the statistical power. This is further affected by the low number of biological replicates generally used in aquaculture studies. To address this issues, this thesis adapted use of the Limma statistical package (Ritchie et al., 2015), normally used in the analysis of microarray data, to the analysis of proteomics data. Limma allows for complex experimental designs including blocking, random effects and batch effects, and allows for the incorporation of sample quality weights. Limma also uses the statistical technique called Empirical Bayes (Smyth, 2004), which increases power for experiments where the number of replicates is typically less than 10. The efficacy of Limma was demonstrated in Chapter 3, with the detection of 14 significantly differentially expressed proteins versus 2 as detected by the application of Perseus statistics (not reported).

Although the main aim of this thesis was to gain physiological insight into key aspects of salmon production, and this has been reached by identifying affected metabolic pathways, a possible limitation is the lack of validation of the results. It is a common practice to compare differences in expressions of a few proteins with significant and different expression patterns in relation to control with the aim of confirming protein identity and the quantification accuracy of the proteomics approach. While immunoblotting remains one of the most common methods of validation, a significant investment is required for quality antibody development, particularly in aquaculture with the high number of species studied and of newly discovered proteins. A superior alternative to immunoblotting is targeted proteomics such as Multiple Reaction Monitoring (MRM), which detects and determines the quantity of a limited set of predefined peptides in a complex mixture of peptides (Parsons and Heazlewood, 2015). The improved genomic annotation and accumulating protein data in the PRIDE database for non-model species such as Atlantic salmon will facilitate the design of marker peptides and the subsequent use of targeted proteomics as validation tool in aquaculture proteomics studies.

In the context of the topics covered in this thesis and of aquaculture research in general, the application of a wider range of proteomics tools and other technologies will provide more detailed and precise insights of the physiological response by the organism under study. Measurement and integration of global sets of biological data from different hierarchical levels of information (DNA, mRNAs, proteins, and metabolites) are the work-horses of modern systems biology approaches (Rho et al., 2008). Proteomics can be combined with other complementary high-throughput "omics" techniques (e.g. genomics, transcriptomics, metabolomics, metagenomics) to produce comprehensive global data that represent different levels of biological information. The clear advantage of such an approach is to increase the power of detecting true causal genes, proteins or metabolites, regulatory networks and pathways, and to obtain a more accurate picture of a given physiological mechanism. To

date, multi-omic data integration has been applied to a vey limited extent in aquaculture research (Hogstrand et al., 2002, De Wit et al., 2008, Morais et al., 2012).

The increasing access to high-resolution mass spectrometry (MS) opens the scope for the analysis of post-translational modifications (PTMs) that are critical to protein function. Phosphorylation and glycosylation are the most well understood PTMs and have been fundamental to biomarker discovery in cancer research (Markiv et al., 2012). The technology and knowledge generated in human medicine can be used to develop the field in aquaculture and in particular in searching biomarkers to monitor disease and stress. So far, the number of aquaculture studies in this field is very limited (Liu et al., 2008, Mezhoud et al., 2008). Improved spectral quality also enables more accurate *de novo* sequencing for identification of novel proteins. This approach derives a peptide sequence without the assistance of a sequence database (Ma and Johnson, 2011) and therefore can overcome the lack of well-curated, 'complete' protein sequence databases for non-model organisms. The application of *de novo* sequencing in aquaculture proteomics is scarce and limited to gel-based approaches (Carrera et al., 2007, Carrera et al., 2010).

An ultimate aim of using proteomics in aquaculture research is to find biomarkers that allow us to make a rapid assessment of changes in the status of fish. While the number of candidate biomarkers in aquaculture research is large, the current limitation for successful biomarker discovery lies in the scarce post-discovery phase verification of these candidates. In this regard, further on-farm verification studies using targeted proteomics are warranted.

## 6.2. CONCLUDING REMARKS

This thesis underlines the usefulness of shotgun proteomics in the area of aquaculture nutrition and stress as a sensitive tool to detect signs of metabolic dysfunctions that correlate to phenotypic traits or alternatively may not been macroscopically visible. This approach has identified potential risks in the dietary use of a high DHA and high ALA oil profile and has raised opportunities for the evaluation of new nutritional strategies to cope with heat stress. These are relevant to the development of salmon aquafeeds in Tasmania, and will also assist global aquaculture in the future. Working within the confines of current technical availability and compared with the field of aquaculture proteomics, this thesis has pushed the limits of protein identification. A new method that effectively achieves extensive proteome coverage in complex protein matrices has been developed, while other experimental considerations (e.g. pooling, statistical approach) that can contribute to improving the accuracy of such methods to estimate protein expression changes in nutritional and stress studies have been highlighted. Candidate biomarkers have been confirmed in this thesis, and new ones have also been proposed, which opens opportunities in the future for on-farm verifications and development of lab-on-chip devices to assess fish status. With the increasing access of aquaculture researchers to high-resolution MS, the maximum value of proteomics towards understanding fish physiology and biomarker discovery will be

obtained by pushing forward the application of bioinformatics resources and the integration with other complementary “omics” technologies.

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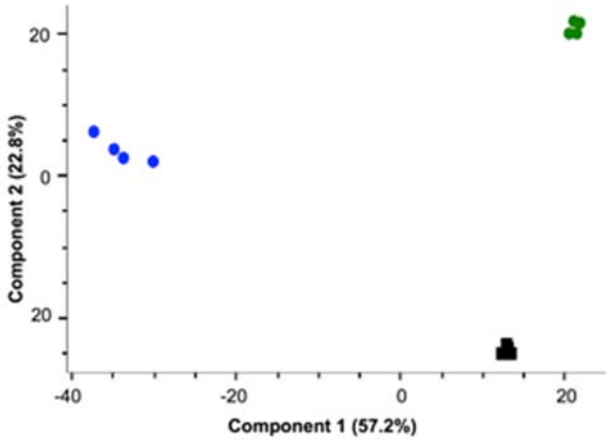
# APPENDIX




**Appendix Figure 2.2 Principal component analysis of the direct extract (black squares), sequential extract SF1 (blue circles) and sequential extract SF2 (green circles) replicates.** High level of consistency is observed across replicates of both protein extraction methods.

**Appendix Table 3.1. Proximate composition (g kg$^{-1}$ DM), total fatty acid (mg g-1 lipid), lipid class (% total fatty acid) and fatty acid composition (% total fatty acid) of commercial feeds**

| | Fry feed[1] | Parr feed[2] |
|---|---|---|
| *Proximate composition* | | |
| Dry matter (g kg$^{-1}$) | 929.6 ± 6.99 | 945.9 ±1.36 |
| Crude protein | 610.1 ± 10.21 | 533.9 ± 4.93 |
| Total lipid | 172.7 ± 3.22 | 170.6 ± 6.09 |
| Ash | 93.2 ± 1.92 | 106.3 ± 0.56 |
| Energy (MJ/kg) | 23.4 ± 0.10 | 23.1 ± 0.08 |
| Total phosphorus[3] | 18.3 ± 0.13 | 13.7 ± 0.02 |
| *Total fatty acid* | 828.3 ± 40.13 | 898.6 ± 18.27 |
| *Lipid class* | | |
| Wax esters | 2.0 ± 0.21 | 1.9 ± 0.34 |
| Triacylglycerides | 74.8 ± 1.59 | 85.4 ± 0.95 |
| Free fatty acids | 3.2 ± 0.82 | 2.8 ± 0.40 |
| Sterols | 2.8 ± 0.40 | 1.7 ± 0.26 |
| Phospholipids | 17.4 ± 1.94 | 8.2 ± 1.14 |
| *Fatty acid composition* | | |
| 14:0 | 5.5 ± 0.27 | 3.8 ± 0.45 |
| 16:0 | 18.1 ± 0.17 | 14.2 ± 0.25 |
| 17:0 | 0.4 ± 0.00 | 0.3 ± 0.00 |
| 18:0 | 3.4 ± 0.05 | 2.5 ± 0.03 |
| Other SFA | 1.2 ± 0.03 | 1.1 ± 0.01 |
| Total SFA | 28.6 ± 0.15 | 21.8 ± 0.69 |
| 16:1n-7 | 7.9 ± 0.46 | 5.0 ± 0.16 |
| 18:1n-7 | 3.1 ± 0.10 | 2.9 ± 0.01 |
| 18:1n-9 | 11.9 ± 0.54 | 21.8 ± 0.22 |
| 20:1n-7 | 0.3 ± 0.01 | 0.2 ± 0.06 |
| 20:1n-9 | 1.2 ± 0.15 | 2.8 ± 1.46 |
| 22:1n-11 | 1.0 ± 0.21 | 7.0 ± 0.25 |
| 24:1n-9 | 0.5 ± 0.01 | 0.8 ± 0.03 |
| Other MUFA | 1.7 ± 0.05 | 0.1 ± 1.35 |
| Total MUFA | 27.7 ± 0.49 | 45.4 ± 0.53 |
| 18:2n-6 | 9.0 ± 1.18 | 8.5 ± 0.12 |
| 20:4n-6 | 0.7 ± 0.02 | 0.4 ± 0.01 |
| Other n-6 | 1.0 ± 0.04 | 0.9 ± 0.11 |
| Total n-6 | 10.6 ± 1.13 | 9.8 ± 0.20 |
| 18:3n-3 | 1.5 ± 0.01 | 2.9 ± 0.01 |
| 18:4n-3 | 2.2 ± 0.11 | 2.0 ± 0.04 |
| 20:4n-3 | 0.7 ± 0.02 | 0.6 ± 0.03 |
| 20:5n-3 | 12.9 ± 0.63 | 7.0 ± 0.24 |
| 22:5n-3 | 1.5 ± 0.06 | 1.0 ± 0.04 |
| 22:6n-3 | 10.1 ± 0.31 | 8.7 ± 0.33 |
| Other n-3 | 0.6 ± 0.03 | 0.3 ± 0.07 |
| Total n-3 | 29.4 ± 1.01 | 22.3 ± 0.62 |
| Other PUFA | 0.2 ± 0.01 | 0.1 ± 0.06 |
| Total PUFA | 40.3 ± 0.34 | 32.3 ± 0.83 |
| n-3:n-6 | 2.9 ± 0.33 | 2.3 ± 0.03 |
| DHA:EPA | 0.8 ± 0.02 | 1.2 ± 0.01 |

Data expressed as mean ± SEM (n = 3)

[1]Fed from 34 to109 dph

[2]Fed from 109 to162 dph

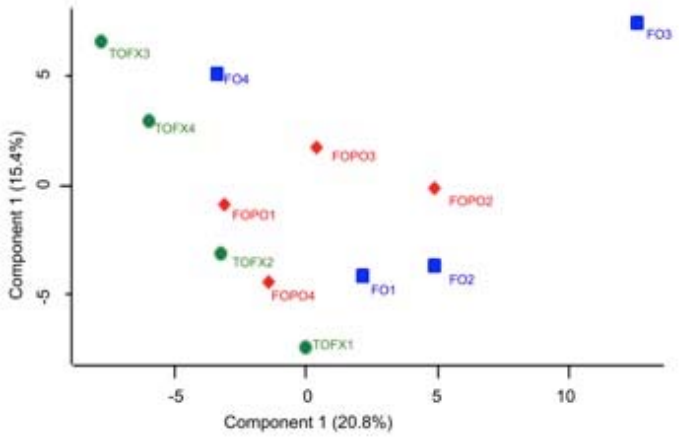
[3]The amount of total phosphorus was calculated based on the total concentration provided by the feed producer.

**Appendix Table 4.1. Apparent digestibility (AD; %) of fatty acids in Atlantic salmon smolt fed FO, FOPO and TOFX diets over a 89 day period**

| | FO | FOPO | TOFX |
|---|---|---|---|
| 14:0 | 94.8 ± 0.90 | 95.9 ± 0.49 | 95.0 ± 0.67 |
| 16:0 | 89.7 ± 0.67 b | 90.1 ± 0.34 ab | 92.6 ± 0.59 a |
| 17:0 | 88.1 ± 0.75 a | 88.3 ± 0.66 a | 69.5 ± 2.17 b |
| 18:0 | 85.0 ± 0.90 | 84.8 ± 0.85 | 88.2 ± 0.87 |
| Total SFA | 90.2 ± 0.71 | 89.6 ± 0.41 | 91.2 ± 0.67 |
| 16:1n-7 | 99.2 ± 0.09 | 99.2 ± 0.13 | 99.2 ± 0.21 |
| 18:1n-7 | 97.7 ± 0.24 | 97.7 ± 0.32 | 98.2 ± 0.29 |
| 18:1n-9 (OA) | 98.1 ± 0.19 b | 98.7 ± 0.20 ab | 99.2 ± 0.20 a |
| 20:1n-9 | 95.8 ± 0.42 | 96.8 ± 0.92 | 95.0 ± 0.81 |
| 22:1n-11 | 95.9 ± 0.53 | 97.1 ± 0.36 | 95.3 ± 0.62 |
| Total MUFA | 97.9 ± 0.20 b | 98.5 ± 0.21 ab | 98.8 ± 0.22 a |
| 18:2n-6 (LA) | 97.7 ± 0.26 b | 99.1 ± 0.12 a | 99.0 ± 0.16 a |
| 18:3n-6 | 99.7 ± 0.15 | 99.6 ± 0.20 | 99.6 ± 0.23 |
| 20:3n-6 | 99.6 ± 0.22 | 98.8 ± 0.68 | 99.2 ± 0.42 |
| 20:4n-6 (ARA) | 98.8 ± 0.15 | 99.0 ± 0.13 | 98.4 ± 0.23 |
| Total n-6 PUFA | 98.2 ± 0.23 b | 99.1 ± 0.15 a | 98.9 ± 0.17 a |
| 18:3n-3 (ALA) | 98.8 ± 0.14 a | 99.1 ± 0.06 a | 96.5 ± 0.43 b |
| 18:4n-3 | 99.7 ± 0.05 | 99.6 ± 0.09 | 99.3 ± 0.19 |
| 20:4n-3 | 99.3 ± 0.15 | 99.0 ± 0.38 | 98.8 ± 0.34 |
| 20:5n-3 (EPA) | 99.6 ± 0.04 | 99.6 ± 0.10 | 99.5 ± 0.09 |
| 22:5n-3 | 99.2 ± 0.11 | 99.0 ± 0.31 | 98.6 ± 0.29 |
| 22:6n-3 (DHA) | 98.9 ± 0.13 a | 98.8 ± 0.18 a | 97.5 ± 0.39 b |
| Total n-3 LC PUFA | 99.3 ± 0.09 a | 99.1 ± 0.16 a | 98.5 ± 0.24 b |
| Total n-3 PUFA | 99.3 ± 0.08 a | 99.2 ± 0.14 a | 98.3 ± 0.21 b |
| Total PUFA | 99.3 ± 0.09 a | 99.2 ± 0.14 a | 98.6 ± 0.19 b |

FO, oil content of feed is 100% fish oil; FOPO, oil content of feed is 20% fish oil and 80% poultry oil; TOFX, oil content of feed is 60% tuna oil and 40% flaxseed oil

Data expressed as mean ± SEM (n=4). Different superscripts within a row denotes significant differences among diets as determined by Tukey-Kramer HSD ($p<0.05$)




**Appendix Figure 4.1. Principal component analysis (PCA) of the liver proteome profiles.** Data points are marked with sample identifiers and replicate number.